CEREAL CHEMISTRY

VOL. XVIII JULY, 1941 No. 4

THE EFFECT OF STORAGE ON THE PROTEIN OF WHEAT, WHITE FLOUR, AND WHOLE WHEAT FLOUR

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(Read at the Annual Meeting, May 1940)

It has been observed on several occasions in this laboratory that not as much protein could be extracted from ground seeds after the meal had stood around for a period of time as from the freshly ground seeds. This decrease in solubility suggested that other fundamental changes in the properties of the proteins may also occur as a result of aging. Because of the meager knowledge available on this subject studies were conducted in order to determine the effects of storage under different conditions on the proteins of several seeds of agricultural importance. This paper presents the results of storage for different intervals during a period of two years on the proteins of wheat kernels, white flour, and whole wheat flour.

Large and increasing quantities of grain are being stored. On July 1, 1940, it was estimated that 270 to 300 million bushels of wheat (new crop) will go under seal. Ten million bushels were in storage on farms under seal, 14 million bushels stored for crop insurance reserve, and 3 million for Commodity Credit Corporation and Federal Surplus Commodities, to be used for relief. At the same time, 400 million bushels of old corn were estimated to be under seal on farms and an additional 65 million bushels stored by the Commodity Credit Corporation. It is important to know the nature and extent of changes that may result during storage and of the effect they may have on the value of the proteins for food and technological purposes.

Large losses are known to be sustained every year through spoilage of grains and other agricultural products as a result of improper storage conditions and methods of handling. Less detectable but highly impor-

tant changes may also occur under apparently satisfactory storage conditions.

It has long been known that wheat and flour undergo certain changes as a result of aging. These changes have concerned primarily the baker and the miller. It is well recognized that flour improves in baking quality during storage for a limited period. It is generally believed that the baking properties of flour, expressed in terms of loaf volume, texture, and other criteria used for denoting baking strength, are intimately associated with, if not dependent upon, the quantity and properties of its gluten—that peculiar complex of the proteins gliadin and glutenin. Heretofore, studies have been confined largely to correlating the effects of storage with changes in baking quality, acidity, diastatic value, viscosity, and other properties. Little data is available on the effect of storage on the fundamental properties of the proteins.

The extensive publications on the effect of storage on the baking qualities of flour have been reviewed by Bailey (1925), and more lately by Cathcart and Killen (1939) in their paper on storage changes in flour.

Information on the nature and extent of the changes that occur in proteins on storage is of importance not only to the baking and milling industry but also in connection with the rapidly increasing production and utilization of proteins for technical purposes. The production yields of the proteins depend largely on their solubility and would be, therefore, largely diminished by denaturation.[1] The suitability of the proteins for the manufacture of specific products such as plastics might well be conditioned by the extent and nature of storage changes. Possible changes in nutritional value are also an important consideration.

Experimental

Preparation of material.—The wheat used in these studies was graded No. 2 Red Winter wheat of the 1937 crop produced in Ohio. It was procured for us through the kind offices of the Milling, Baking, and Chemical Laboratory of the Grain Division of the Bureau of Agricultural Economics, United States Department of Agriculture, and was milled during the week of November 15, 1937, in their experimental flour mill. The wheat was cleaned in an experimental milling separator and scourer. The loss in preparation for milling was approximately 3.5%. The wheat was milled in the usual way by tempering at room temperature in two stages to 15%, and the milling was done without generation of heat in any way.

In the preparation of the whole wheat flour, in order to avoid development of heat during grinding, the wheat was coarsely cracked in

[1] The term *denaturation* as used in this paper refers only to decrease in solubility of the proteins.

an attrition mill, and the product was then reduced to a fine powder by reduction on the smooth rolls of the flouring mill.

The white flour was what is known as a 95% patent flour. In other words, all the flour possible was removed from the wheat, and the last 5%, which was discolored with germ stock and bran, was discarded. On a percentage extraction basis the wheat yielded 70% of flour.

Storage of material.—On November 24, 1937, samples of the white flour and of the whole wheat flour were placed in sealed Mason jars and in closely woven cotton bags. The samples were stored in sealed cupboards in a room with controlled temperature and humidity. The temperature was maintained at about 76° ± 3° or 4°F. and the relative humidity at about 55%. Samples in sealed jars and in closely woven cotton bags were also stored in a refrigerating room maintained at a temperature of 30°F. Each jar contained approximately enough material to provide for the required analyses at the end of each storage period, thus making it unnecessary to open any jar before the end of the stated storage periods. Samples also of the whole kernels were stored in half-gallon glass jars at 76°F. and 30°F.

Analyses and procedure.—Analyses of the white flour and whole wheat flour samples stored in jars were made at the end of 1, 4, 7, 12, and 24 months of storage, and of those stored in bags at the end of 7, 12, and 24 months. The whole seeds, ground to the approximate fineness of the original whole wheat flour, were analyzed after storage periods of 9 and 24 months.

The different analytical determinations made on the samples were considered suitable to reveal the nature and extent of some of the fundamental changes that may occur in the properties of the proteins during varying periods and conditions of storage. All the determinations were made in duplicate or triplicate, and the results given are averages of closely agreeing values.

The first series of analyses were made on the samples at the time they were placed in storage. The results of these analyses serve as a basis of comparison with similar data obtained later on the stored samples whereby the extent of the changes that occurred may be ascertained. Throughout the investigation special care was taken to have all the corresponding analyses made in the same way and under the same conditions in order to have the results of the different sets of analyses on as comparable a basis as possible. For the analyses of the stored wheat kernels the samples were ground to a fine powder in a hand-driven mill, care being taken to have the material reduced to the same degree of fineness for each set of analyses.

Moisture and total nitrogen.—Determinations were made on the freshly prepared material, and on all samples at the end of each storage

period. The moisture was determined according to the official A.O.A.C. method (1940), by heating about 2 g. of the samples at 130°C. for one hour. Nitrogen was determined in triplicate on 1-g. samples by the Kjeldahl method and the average results calculated to a moisture-free basis.

Free ammonia nitrogen.—Duplicate samples of the meals corresponding to 10 g. of the moisture-free material were transferred to two Claissen flasks (1.5 to 2 liters capacity) and to each flask were added 200 ml. of distilled water and 100 ml. of alcohol. About 8-ml. portions of *n*-butyl alcohol were added in order to prevent excessive foaming. The mixtures were then made slightly alkaline by addition of a 10% suspension of $Ca(OH)_2$, and subjected to distillation under reduced pressure (20 to 30 mm.) for 45 minutes at 40°C. to 50°C. The free ammonia was collected and determined in 12 to 20 ml. tenth-normal H_2SO_4 according to the method of Van Slyke (1911–1912) for amide nitrogen determination.

Free fatty acids.—Determinations were made only on the whole wheat flour and on the wheat kernels. The kernels were first ground to a fine powder in a small hand mill. The oil was extracted with ether from 100-g. samples of the material in a special extraction apparatus, and the ether was removed by evaporation on a steam bath. The residual oil was dissolved in about 20 ml. of absolute alcohol. The alcohol was removed by evaporation. The residual oil was weighed and dissolved in 30 ml. of alcohol, which had been made neutral to phenolphthalein, and the solution was titrated with tenth-normal NaOH. The free fatty acid content is calculated in terms of oleic acid according to the formula: 0.282 × ml. tenth-normal alkali × 100/grams oil extracted.

True protein.—As a measure of the extent to which degradation of the proteins occurred during the storage periods, determinations were made of the so-called "true protein value" (protein nitrogen). The determinations were made in duplicate on samples equivalent to 1 g. of the moisture-free material according to the copper hydroxide method of Stutzer (1881).

Soluble nitrogen.—Extractions of the meals and flours were made with NaCl, alcohol, and sodium salicylate. In order that all extractions of the fresh and stored samples with a given solvent could be conducted to give strictly comparable results, special care was taken to have them carried out under as uniform conditions as possible.

The NaCl extractions were conducted in the temperature-controlled room at 76°F. Ten-gram samples of the material, which had been allowed to acquire room temperature before starting the extractions, were shaken at a uniform rate in a mechanical shaker for one hour with 200 ml. of 3% NaCl solution. After centrifugation, the residue was again

similarly extracted with 50 ml. of the extractant.[2] The combined extracts were transferred to a 300-ml. graduated flask and made to volume with the salt solution.

Extractions were made also with 3% sodium salicylate solution in the same manner as described for the NaCl extractions.

In view of the fact that a large proportion of wheat protein is soluble in dilute alcohol, 10-g. samples of the material were extracted for one-half hour with 200 ml. of 70% alcohol at room temperature. The clear extract was made up to volume in a 200-ml. flask.

Total nitrogen was determined in aliquots of the extracts.

Amino nitrogen.—Amino nitrogen determinations were made in duplicate on 10-ml. aliquots of the fresh NaCl extracts according to the Van Slyke method. An increase in amino nitrogen is a criterion of proteolysis.

Nitrogen precipitable by trichloracetic acid.—Inasmuch as trichloracetic acid precipitates the whole protein molecule, but does not precipitate its smaller hydrolytic products, the results obtained by this determination can also be taken as a measure of the extent of protein degradation that occurred during storage.

The nitrogen precipitable by trichloracetic acid was determined in 25-ml. aliquots of the original NaCl and sodium salicylate extracts already referred to. The quantities of 15% trichloracetic acid required to give the maximum precipitation in the white flour and the whole wheat extracts were first determined in the extracts of the fresh, unstored materials, and these quantities were subsequently used in the determinations at the end of the different storage periods. The NaCl extracts of the white flour required 6 ml. of the precipitant per 25 ml. of the extract, and 5 ml. was used for the whole wheat flour extracts. In the case of the sodium salicylate extracts, 6 ml. of the precipitant was added per 25-ml. aliquot of both the white flour and the whole wheat flour extracts.

The trichloracetic acid precipitates were separated by centrifugation. Nitrogen was determined in aliquots of the filtered supernatant liquids, and that in the precipitates was calculated by difference.

Digestibility.—The amount of nitrogen rendered soluble by digestion of the samples with pepsin and trypsin under strictly comparable conditions was used to follow changes in digestibility of the protein as a result of storage.

A quantity of the flour equivalent to 30 g. of the moisture-free material and 2 g. of pepsin was mixed with tenth-normal HCl to a volume of 600 ml. The mixture, in a liter Erlenmeyer flask, was incubated for

[2] In carrying out the extractions of the meals it was not attempted to make them exhaustive because comparative data only were desired.

TABLE I

EFFECT OF STORAGE ON THE PROTEINS OF WHITE FLOUR

(Results expressed in milligrams per 100 grams flour)

Determinations	Fresh material	Stored in closed jars										Stored in bags					
		Months at 30° F.					Months at 76° F.					Months at 30° F.			Months at 76° F.		
		1	4	7	12	24	1	4	7	12	24	7	12	24	7	12	24
		mg. per 100 g. flour										*mg. per 100 g. flour*					
Moisture	12,900	12,900	12,900	12,900	12,900	14,020[1]	12,900	12,900	12,900	12,900	12,990	14,300	14,300	14,310	11,300	11,300	11,340
Total nitrogen	1,910	1,900	1,890	1,900	1,900	1,890	1,910	1,890	19,100	1,900	1,900	1,900	1,900	1,880	1,890	1,890	1,890
True protein nitrogen	1,284	1,271	1,239	1,184	1,142	1,069	1,212	1,146	1,106	1,044	989	1,142	1,121	1,048	1,086	1,012	955
Free ammonia nitrogen	23.5	24.1	23.8	24.1	24.5	24.0	24.1	23.8	23.7	24.1	24.0	24.5	24.1	24.0	24.2	24.2	23.0
Nitrogen soluble in 3% NaCl	624	431	410	410	399	392	357	337	326	315	268	409	394	376	305	284	243
Nitrogen soluble in 70% alcohol	1,026	938	924	924	903	884	882	832	812	777	713	917	900	861	791	735	683
Nitrogen soluble in 3% sodium salicylate	—	819	787	777	756	726	798	734	725	612	611	767	725	687	704	609	551
Nitrogen in NaCl extracts precipitable by trichloracetic acid	507	339	299	273	247	227	264	219	182	152	97	246	217	190	148	108	64
Nitrogen in sodium salicylate extracts precipitable by trichloracetic acid	—	793	749	719	685	651	759	717	640	575	507	695	640	596	600	499	437
Amino nitrogen in NaCl extracts	59.6	51.6	50.7	59.5	64.5	67.0	74.8	71.7	74.4	76.7	75.0	61.9	68.6	71.0	78.3	79.5	83.0
Digestibility	1,894	1,870	1,819	1,796	1,738	1,662	1,815	1,781	1,760	1,691	1,573	1,767	1,724	1,631	1,731	1,681	1,524

[1] High moisture caused by loosened cover.

72 hours at 38°C. At the end of the period the digestion mixture was brought to pH 8–9 by addition of 16 ml. of fifth-normal NaOH. Two grams of trypsin [3] was then added and the digestion was continued for 72 hours. During both the peptic and tryptic digestions the digests were covered with a layer of toluene as a preservative, and the mouths of the flasks were plugged with cotton. At the end of the tryptic digestion the mixture was filtered on folded filters. Ten-milliliter aliquots of the filtrates were taken for total nitrogen determinations. Corrections were made for the nitrogen in the enzymes used.

Discussion of Results

Throughout the two years' storage period all the samples remained free from any indication of insect infestation or mold. There was no noticeable change in color or odor in the material stored in Mason jars, nor in the white flour stored in bags at 76°F. The whole wheat flour stored in bags at 76°F. had developed by the end of two years a slightly rancid odor. The white flour and the whole wheat flour stored in bags in the cold storage room had acquired a slight off-odor due to odors absorbed from other material stored in the room.

Tables I, II, and III show, respectively, the results of analyses of white flour, whole wheat flour, and wheat kernels made at the time they were placed in storage, and at the end of the storage intervals. These results are expressed as milligrams per 100 g. of the samples stored, and are calculated to a moisture-free basis. For convenience in visualizing the extent of the changes, and for the sake of comparison, the changes in the proteins of the white flour and of whole wheat proteins are summarized in Table IV. The changes are expressed as percentage decreases from the values obtained for the controls or fresh material. The percentages have been calculated from the data given in Tables I, II, and III. In order to save space, percentage decreases during only the first and twenty-fourth months are shown.

The results show that marked alterations occurred in the proteins of the samples during storage. These changes are manifested by decreases in solubility, in true protein content, in amount of protein precipitable by trichloracetic acid, and in digestibility. The amino nitrogen content increased. The extent of the changes depends on temperature, type of containers, duration of storage, and nature of the material stored. Samples stored at 76°F. were affected much more than those stored at 30°F., and those in bags more than those in sealed jars. The changes in the white flour were in general much greater than those in the whole

[3] Fairchild Brothers and Foster's commercial preparations of pepsin and trypsin were used for the digestions.

TABLE II

EFFECT OF STORAGE ON THE PROTEINS OF WHOLE WHEAT FLOUR

(Results expressed in milligrams per 100 grams flour)

Determinations	Fresh material	Stored in closed jars										Stored in bags					
		Months at 30° F.					Months at 76° F.					Months at 30° F.			Months at 76° F.		
		1	4	7	12	24	1	4	7	12	24	7	12	24	7	12	24
		mg. per 100 g. flour										*mg. per 100 g. flour*					
Moisture	10,950	10,960	10,950	10,960	10,960	10,980	10,950	10,950	10,940	10,950	10,960	14,080	14,090	14,110	10,490	10,490	10,510
Total nitrogen	2,140	2,130	2,140	2,130	2,130	2,130	2,150	2,140	2,130	2,130	2,130	2,130	2,120	2,120	2,130	2,130	2,140
True protein nitrogen	1,682	1,635	1,608	1,569	1,534	1,507	1,583	1,512	1,450	1,401	1,361	1,534	1,506	1,425	1,436	1,366	1,285
Free ammonia nitrogen	37.0	37.0	37.0	37.0	38.0	37.0	37.0	37.0	37.0	37.0	37.0	36.0	37.0	37.0	37.0	37.0	38.0
Nitrogen soluble in 3% NaCl	756	641	587	583	572	550	588	546	536	520	477	573	546	515	509	489	428
Nitrogen soluble in 70% alcohol	910	742	713	707	700	689	714	679	658	637	583	686	672	653	644	595	538
Nitrogen soluble in 3% sodium salicylate	—	1,103	1,059	1,019	998	961	1,103	1,008	977	954	894	998	956	905	956	893	866
Nitrogen in NaCl extracts precipitable by trichloracetic acid	616	502	411	388	364	326	450	351	303	262	213	371	325	278	251	205	138
Nitrogen in sodium salicylate extracts precipitable by trichloracetic acid	—	999	943	869	829	781	1,025	865	795	746	675	842	780	709	767	678	627
Amino nitrogen in NaCl extracts	109.0	94.16	92.8	100.0	108.0	109.0	125.0	122.0	128.0	129.0	130.0	111.0	117.0	117.0	136.0	139.0	137.0
Digestibility	2,050	2,035	2,046	2,026	1,968	1,887	2,093	2,076	2,026	1,961	1,857	1,954	1,911	1,788	1,940	1,861	1,751

wheat flour. Significant changes occurred also in the wheat kernels, although not so pronounced as in the flours. Doubtless even greater changes than those here recorded would have been indicated had it been possible to analyze the flours immediately after milling. Instead, there was an interval of about 10 days between the time of milling and that of the first analyses of the material. The changes that occurred during the first month of storage were in some instances as much as three-fourths of that found at the end of two years' storage.

Moisture.—The moisture content of the samples remained practically constant throughout the entire storage period. The relatively constant temperature and humidity under which the materials were stored did not allow much change in moisture content.

Total nitrogen.—There was no significant change in the nitrogen content of the samples. This was to be expected, inasmuch as there were no indications of gross spoilage. A decrease in nitrogen content would require a much more drastic and far-reaching decomposition of the protein than would be likely to occur as a result of enzymatic or oxidative effects under the conditions to which the materials used in this study were stored.

Free ammonia.—The values found for free ammonia nitrogen were also practically constant at all intervals of the storage period.

Solubility changes.—Two striking types of change are revealed by the results of the storage studies. One consists of a denaturation of the proteins whereby they became progressively less soluble as the length of time of storage increased. The other change involves a breaking down of the protein-yielding products having properties that are not characteristic of native or intact protein, such as precipitability with trichloracetic acid and behavior toward the Stutzer method used for determining true protein. The extent of denaturation was followed by determination of solubility in 3% NaCl solution, 70% alcohol, and 3% sodium salicylate.

The greatest decrease was found in solubility of the proteins of white flour in NaCl. When stored in a bag at 76°F. for two years the percentage decrease amounted to 61%, and 57% after similar storage in a Mason jar. The rapid rate of denaturation is also notable. Stored in a jar under the same conditions the decrease at the end of the first month was 43% or three-fourths as much as at the end of two years. Even storage at 30°F. resulted in denaturation to a noteworthy degree. After storage in a jar at 30°C. for 24 months the solubility of the white flour proteins had decreased 37% of that of the fresh flour, and 40% after storage in a bag at the same temperature.

Solubility of the white flour proteins in 70% alcohol also decreased with increasing time of storage, but only to about one-half the extent it did in NaCl. After 24 months' storage in a bag at 76°F. the amount of

TABLE III

EFFECT OF STORAGE ON THE PROTEINS OF WHEAT KERNELS
(Results expressed in milligrams per 100 grams wheat)

Determinations	Fresh material	Stored in jars			
		Months at 30° F.		Months at 76° F.	
		9	24	9	24
Moisture	10,950	10,960	10,960	10,950	10,940
Total nitrogen	2,140	2,140	2,140	2,140	2,140
True protein nitrogen	1,682	1,653	1,574	1,632	1,519
Free ammonia nitrogen	37.0	37.0	37.0	38.0	38.0
Nitrogen soluble in 3% NaCl	756	620	606	557	516
Nitrogen soluble in 70% alcohol	910	770	748	722	713
Nitrogen soluble in 3% sodium salicylate	—	1,061	1,038	998	922
Nitrogen in NaCl precipitable by trichloracetic acid	616	518	509	134	163
Nitrogen in sodium salicylate precipitable by trichloracetic acid	—	943	919	874	788
Amino nitrogen in NaCl extracts	109.0	102.0	100.0	94.0	88.0
Nitrogen soluble in peptic-tryptic digests	2,050	2,033	1,987	2,026	1,878

nitrogen extractable with alcohol had decreased 36% of that extractable from the flour when fresh, and 19% after a similar storage at 30°F. When stored in jars the corresponding decreases in solubility were 33% and 17%, respectively. The solubility in alcohol decreased much more rapidly during the early part of the storage than later. After one month's storage in a jar at 76°F. the solubility had decreased 17%, or about one-half as much as it did during storage for 24 months.

When sodium salicylate was used as an extractant, the same decreasing trend in solubility of the proteins of the white flour on storage was shown as when NaCl and alcohol were used. Unfortunately, sodium salicylate was not used on the fresh material. The first extractions were made on the flour samples in jars after they had been stored for one month, and after seven months on the samples stored in bags. Doubtless denaturation had already occurred to a large extent before the first extractions were made.

A comparison of the solubility changes in the white flour with those in the whole wheat flour under similar conditions of storage brings out several interesting facts. The effect of storage upon the solubility of the white flour proteins, as measured by the NaCl-extractable nitrogen, was consistently much greater than the effect upon the proteins of whole wheat flour. The decreases in solubility ranged from one and one-half times to twice as much for the white flour as for the whole wheat flour. If we assume that the denaturation of the proteins on storage is an oxidizing effect, and there is reason to believe that it is, the difference in

TABLE IV

EFFECT OF STORAGE ON THE PROTEINS OF WHITE FLOUR, WHOLE WHEAT FLOUR AND WHEAT KERNELS

(Results expressed in percentage decreases)

Determinations	Months at 30° F.		Months at 76° F.	
	1	24	1	24
WHITE FLOUR STORED IN JARS				
	%	%	%	%
True protein nitrogen	1	17	5	23
Nitrogen soluble in 3% NaCl	31	37	43	57
Nitrogen soluble in 70% alcohol	12	17	17	33
Digestibility	1.3	12.0	4.0	17.0
WHOLE WHEAT FLOUR STORED IN JARS				
True protein nitrogen	3	10	6	19
Nitrogen soluble in 3% NaCl	15	27	22	37
Nitrogen soluble in 70% alcohol	18	24	21	36
Digestibility	1.0	7.0	2.0	9.0
WHEAT KERNELS STORED IN JARS				
	9	24	9	24
True protein nitrogen	1.7	6.4	2.9	9.69
Nitrogen soluble in 3% NaCl	18.0	19.84	26.3	31.7
Nitrogen soluble in 70% alcohol	15.4	17.8	20.6	21.6
Digestibility	0.83	3.07	1.66	8.39
WHITE FLOUR STORED IN BAGS				
	7	24	7	24
True protein nitrogen	11	18	15	26
Nitrogen soluble in 3% NaCl	34	40	51	61
Nitrogen soluble in 70% alcohol	14	19	26	36
Digestibility	7.0	14.0	9.0	20.0
WHOLE WHEAT FLOUR STORED IN BAGS				
True protein nitrogen	9	15	15	24
Nitrogen soluble in 3% NaCl	24	32	33	43
Nitrogen soluble in 70% alcohol	27	28	29	41
Digestibility	5.0	13.0	5.0	15.0

the solubility behavior of the proteins of the two flours toward NaCl may be quite satisfactorily explained. Whole wheat flour contains to a much greater extent than white flour a number of constituents which, because of their antioxidant properties, may well protect the proteins from oxidative denaturation. These substances, residing chiefly in the wheat embryo and in the bran, represent phosphatides, unsaturated fatty acids, sterols, glutathione, and other substances that are very susceptible to oxidation. According to Rewald (1936), 80% of the phosphatides of wheat germ oil is of the lecithin type and 20% of the cephalin type. The antioxidant property of lecithin has been shown by Evans (1935) to be almost as great as that of KCN. Oils to which as little as 0.5% lecithin had been added withstood oxidation for four months at room temperature. Cephalin is readily oxidized by atmospheric oxygen at ordinary temperature (Page and Bülow, 1931). According to Sullivan and Bailey (1936a), the unsaturated fatty acids of wheat germ oil amount to 84% of the total fatty acids, and consist of α- and β-linolenic acids (3.55%), α- and β-linolic acids (52.31%), and oleic acid (28.14% by difference). Furthermore, wheat is known to contain several unsaturated sterols, the α_1- and α_2-sitosterols, and dihydroxysitosterol, in which the bran and the embryo are particularly rich. Approximately 70% of the unsaponifiable fraction of wheat embryo consists of a mixture of sterols (Sullivan and Bailey, 1936b). In addition to the sitosterols and dihydroxysitosterol there is evidence showing the presence of another unsaturated sterol with at least two double bonds. The presence of polyene hydrocarbons was also indicated. That fat constituents may be an important factor in protecting the proteins from denaturation is further supported by the fact that fat-free, solvent-extracted soybean meal shows a greater decrease in NaCl extractable nitrogen than meal containing 11% of fat.[4]

When the extent of denaturation of the proteins of the two flours is measured by the amount of nitrogen extracted by 70% alcohol, we find that the order is reversed from what it is when NaCl is used. Extraction with alcohol showed that denaturation occurs consistently to a greater extent in the proteins of whole wheat flour than in the white flour. In order to seek an explanation of this difference in behavior with respect to the two extractants, consideration must be given to the differences in the protein composition of the different parts of the wheat kernel. The white flour is derived almost entirely from the endosperm. Its proteins consist chiefly in about equal quantities of gliadin, the alcohol-soluble protein, and glutenin, a protein which is insoluble in water, neutral salt solutions, or alcohol, but is soluble in dilute acids or alkalies.

[4] Unpublished results obtained in this laboratory.

The proteins of the endosperm comprise about 73% of the total seed protein. In addition to the endosperm, the whole wheat flour includes the bran and the embryo which represent, respectively, 22% and 4.5% of the total seed protein. The proteins of the bran (Jones and Gersdorff, 1923) consist of an alcohol-soluble protein, a globulin, and an albumin. The alcohol-soluble protein predominates. The embryo proteins consist of a globulin, albumin, and a proteose (Osborne and Mendel, 1919). Undoubtedly most of the protein extracted from white flour by alcohol represents gliadin.

The values given in Table I show that alcohol extracted 56% of the total nitrogen of the white flour, which corresponds roughly to the gliadin nitrogen. In the case of the whole wheat flour, the quantity of total nitrogen extracted was greater than that extracted from the white flour because of the higher nitrogen content of the whole wheat flour. The percentage of the total nitrogen extracted (42%) was considerably less, however. In addition to the gliadin and glutenin of the endosperm, the whole wheat contains the proteins of the bran and embryo. It appears that some, at least, of these additional proteins denatured more readily than the gliadin.

That gliadin denatured to a much less extent than other proteins of wheat is also shown (Table IV) by the fact that the percentage decrease in solubility of the white flour proteins at the end of successive storage intervals was found to be approximately twice as much in NaCl as in alcohol.

A comparison of the solubility of the proteins of whole wheat flour in NaCl with that in alcohol after different periods of storage shows consistently a little greater denaturation when measured by the NaCl extractable nitrogen, but the difference is not very significant.

Protein degradation.—The determinations of true protein nitrogen, nitrogen precipitable by trichloracetic acid, and amino nitrogen were designed to show whether any degradation of the proteins occurred on storage.

In the true-protein determinations the insoluble copper compound of the coagulated protein represents the unchanged native protein. The degradation products, such as peptides, proteoses, and amino acids, remain in the filtrates. The results show consistently decreasing values in the true protein nitrogen content of the flours as the storage intervals increased. The decreases were significantly greater in the white flour than in the whole wheat flour, and greatest in the samples stored in bags at 76°F. Even at 30°F. the white flour stored in jars for 24 months showed a percentage decrease of 17%. When stored in bags at 76°F. the decrease amounted to 26%. These decreases show definitely that the protein had undergone some kind of breakdown during storage.

Trichloracetic acid precipitations made on the NaCl extracts of the stored samples also show a breakdown of the proteins, increasing with the length of the storage periods. It is emphasized, however, that the figures indicate a greater extent of protein breakdown during storage than actually occurred. Consideration must be given to the diminishing amounts of protein present in the NaCl extracts with increasing storage periods as a result of denaturation of the proteins in the flour. Thus, for example, in the case of the white flour stored in a bag at 76°F. for two years the amount of nitrogen precipitated by trichloracetic acid decreased from 507 mg. to 64 mg., a drop representing 87%. If, however, the decrease is calculated on the amount of nitrogen remaining in the NaCl extract after precipitation with trichloracetic acid rather than on the amount precipitated, thus largely avoiding in the calculation the complicating factor of denaturation, the much smaller decrease of 53% is indicated.

The amounts of amino nitrogen found in the NaCl extracts of the flours show a definitely increasing trend with the length of storage periods. At the end of 24 months the amino nitrogen found in the salt extracts of the white flour stored in jars at 30°F. had increased from 59.6 mg. to 67 mg., and to 75 mg. when stored at 76°. The sample stored for the same periods in a bag at 30°F. showed an increase of amino nitrogen to 71 mg., and to 83 mg. when stored at 76°F. The corresponding increases for whole wheat flour were not as great, particularly at low-temperature storage. At 76°F., however, the sample stored in a bag showed an amino nitrogen increase at the end of 24 months from 109 mg. to 137 mg. For the reason given below the actual increases in amino nitrogen must be materially greater than the amounts found in the extracts.

As in the case of the trichloracetic acid precipitations, an exact evaluation of the amino nitrogen results obtained is complicated by the fact that the NaCl extracts of the samples after each successive storage period contained decreasing amounts of protein. It has been shown that proteins yield amino nitrogen in amounts corresponding very closely to one-half of their lysine nitrogen; consequently there should be added to the amounts of amino nitrogen actually found in the extracts of the stored samples the amounts of amino nitrogen that would have been yielded by the proteins rendered insoluble during the storage periods. The results would then show materially larger increases of amino nitrogen than those actually determined (Table I). No data, however, are available on the exact amount of amino nitrogen represented by the denatured protein fractions. An approximation can be made by assuming that the free amino nitrogen of the denatured proteins represents 5% of their total nitrogen. This assumption is based on the fact that

certain proteins, such as casein and hemoglobin, yield from 5% to 6% of free amino nitrogen (Van Slyke and Birchard, 1913–1914). It is realized that 5% may be somewhat too high for the white flour because of the low lysine content of gliadin. On the other hand, glutenin, the other protein of white flour, contains a relatively large amount of lysine.

If the data for amino nitrogen in the NaCl extracts shown in Table I were recalculated on the basis outlined above, the results would indicate that there is a very material increase in the amino nitrogen of the samples as a result of storage. The amount of nitrogen rendered insoluble by the end of any given storage period is equal to the total nitrogen in the extract minus the total nitrogen found in the salt extract of the fresh material. Five percent of this nitrogen added to the amount of amino nitrogen actually found in the NaCl extracts will give a more nearly accurate figure for amino nitrogen increase. As an illustration, the amino nitrogen found in the NaCl extract of white flour stored for 24 months in a bag at 76°F. calculated on the basis above outlined becomes 102 mg. instead of 83 mg., which represents an increase of 71% as compared with the amino nitrogen in the salt extract of the fresh material.

It is of interest to point out that there is an apparent inconsistency in the amino nitrogen values determined at the end of one and four months' storage (Table I). The amounts of amino nitrogen actually found in the salt extracts were less at the end of the first and the fourth month than that of the fresh material. These apparent decreases are accounted for by the higher rate of denaturation of the proteins during the first few months than subsequently, and they disappear when the values have been corrected for the amino nitrogen in the denatured fractions.

Digestibility.—The data presented for the digestibility values represent the amount of soluble nitrogen found in the peptic-tryptic digests of the samples at the end of the various storage periods.

The results of the digestibility determinations indicate a definitely decreasing trend in the digestibility of the proteins as the periods of storage increased. The decreases in digestibility were greater in the white flour than in the whole wheat flour, and least in the kernels. The decreases during the first seven months, however, were small compared with the changes already noted in solubility, true protein value, and precipitability with trichloracetic acid for the same periods. At the end of 24 months the white flour stored in bags at 76°F. showed a decrease of 20% compared with 15% for whole wheat flour. Wheat kernels stored in jars for 24 months showed a digestibility decrease of 8%. For some reason that we cannot explain, there was a slight increase in

the values found for whole wheat flour stored in jars at 76°F. at the end of the first and fourth months.

Free fatty acids.—It is well known that on aging cereal flours develop increasing amounts of free fatty acids. Zeleny and Coleman (1938, 1939) recently have contributed to our knowledge in this field, and have surveyed the literature on the subject.

Kozmin (1935) and Barton-Wright (1938) reported that deterioration of gluten quality during storage is to be attributed largely to the effect of accumulating quantities of free fatty acids.

In order to get some collateral data that might be of value in explaining the nature of the causative agents that bring about the changes in the proteins during storage, determinations were made also on the free fatty acid content of the samples at the end of the storage intervals. Because of the small quantities of fat in the white flour, these determinations were made only on the whole wheat flour and on the kernels.

The rate of increase in free fatty acids in the whole wheat flour during storage varied with the temperature, type of container, and duration of storage up to the end of 12 months. There was not much increase during the second year. At the time the flour was placed in storage the fatty acid content amounted to 7% of the extracted oil. After storage for 12 months in a jar at 30°F. the value had risen to 13%, and to 35% when stored at 76°F. When stored in bags under otherwise the same conditions the values had increased to 15% and 36%, respectively. The wheat kernels gave a value of 12% and 15% after storage in jars for two years at 30° and 76°F., respectively.

Possible Causes of Storage Changes

The question naturally arises as to what may be the agents that are accountable for the protein changes observed. Are they attributable to enzymatic, oxidative, or other effects? That they were due to the action of molds or insects seems to be pretty well excluded. We have no reason to believe that they can be explained by bacterial action. The degradation of the proteins as manifested by increase in amino nitrogen, decrease in true protein, and decrease in the amount of protein precipitated by trichloracetic acid, we believe is best explained by the action of enzymes. It certainly seems that the increase in amino nitrogen can be accounted for only by enzymatic effect whereby cleavage of the protein occurs at the peptide linkages. It is known that wheat contains enzymes that attack proteins. Balls and Hale (1935, 1938) identified the proteinase of whole wheat and wheat bran as belonging to the papain type of enzymes. More recently, Hale (1939) extracted a proteinase from patent flour that appeared to be the same as that in wheat bran. The proteinase

showed a definite, but not extensive, proteolytic activity, indicating that it may be accompanied by a peptidase. Its effect is regarded as a "protein modification rather than extended hydrolysis."

Decrease in solubility of the proteins might be ascribed, at first thought, to the effect of free fatty acids. This explanation, however, finds no support in the fact that denaturation as measured by solubility in NaCl occurred to a greater extent in white flour than in whole wheat flour which contained much more fatty acids. The most plausible explanation is that which has been already discussed on another page, namely, that the denaturation was the result of oxidation, occurring spontaneously or as the effect of oxidases present in the flours. Further work is planned in order to get more evidence on this question. Feeding experiments are in progress to study the effect of storage on the nutritional value of the proteins.

Summary

The effect of storage under different conditions upon the proteins of white flour, whole wheat flour, and wheat kernels has been studied at various intervals over a period of two years. The results show that three different types of alterations occur: (1) a decrease in the solubility of the proteins, (2) a partial breakdown of the proteins indicated by decrease in true protein content, by decrease in the amount of nitrogen precipitable by trichloracetic acid, and by increase in amino nitrogen, and (3) decrease in digestibility.

The extent of the changes depends on temperature, type of containers, duration of storage, and the nature of the material stored. Samples stored at 76°F. were affected more than those stored at 30°F., and those in bags more than those in sealed jars. Changes in white flour were in general greater than in whole wheat flour. Significant changes occurred also in the wheat kernels, although not as great as in the flours. The total nitrogen and free ammonia remained unchanged. The extent of denaturation of the proteins was followed by determination of solubility in 3% NaCl, 70% alcohol, and 3% sodium salicylate. The greatest decrease was found in solubility of the proteins of white flour in NaCl. When stored in a bag at 76°F. for two years the percentage decrease amounted to 61%, and 57% after similar storage in a sealed glass jar. The alteration of the proteins is ascribed to the effect of enzymes and oxidation.

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OBSERVATIONS ON THE CHARACTER OF RECORDING-DOUGH-MIXER CURVES ON FLOURS DILUTED WITH WHEAT STARCH

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(Received for publication October 31, 1940)

Effort toward a determination of the correlation between physical properties of doughs and baking performance has been continued during the past decade. A number of people have done a considerable amount of work but comparatively little has been published.

One of the obstacles in comparing flours is the variation resulting from differences of protein level. Larmour, Working, and Ofelt (1939) showed that recording dough-mixer curves for any given variety varied in character at different protein levels and that the height of the curve increased with increasingly higher protein content. In order to minimize differences in protein quantity as a factor in studying baking properties of wheat flours, Sandstedt and Ofelt (1940) used flours diluted with starch to a common protein level. This suggested that the mixing characteristics of flours also should be compared on a common protein basis. An investigation was made of the effects of dilution, by the addition of wheat starch, on a number of flours. This paper is a report of the observations made.

Methods and Materials

The wheat starch used for dilution purposes was prepared according to the technique used by Sandstedt, Jolitz, and Blish (1939), discarding the "amylo-dextrin" fraction.

The several variety-protein series of flours used by Sandstedt and Ofelt (1940) for the study of baking properties were also used in this investigation. These samples were obtained from the Department of Milling Industry at Kansas State College, through the courtesy of Dr. E. G. Bayfield and were similar to the series used by Larmour, Working, and Ofelt (1939) in their investigations on quality of hard winter wheats. The southwestern composite was made up of experimentally milled flours from 18 southwestern wheats. The Cheyenne selection was obtained from the Department of Agronomy of the University of Nebraska, and the Chiefkan was a field sample.

A National Micro Recording Mixer was used and each curve represents 35 g. of flour on a 15% moisture basis, with normal baking absorption. Absorption was determined on each of a number of normal flours and on definite dilutions of these flours with wheat starch.

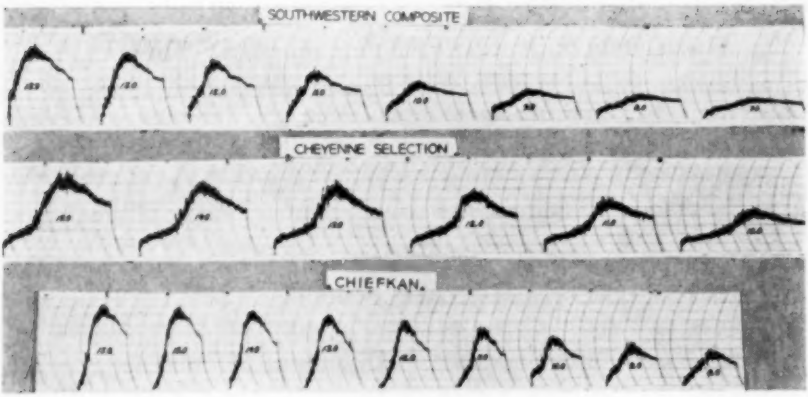
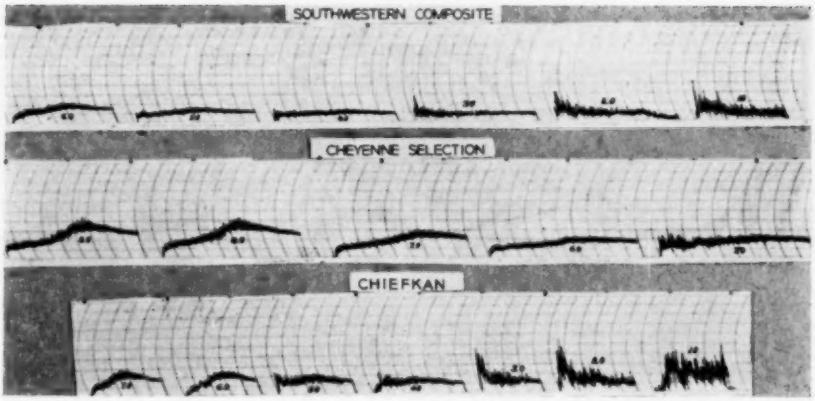


Fig. 1. Recording-micro-mixer curves that show the effects on curve character obtained by diluting with wheat starch to different protein levels. The curve on the extreme left in each series is

From information obtained in this manner it was possible to calculate the absorption attributable to the starch and thus to calculate the proper absorption for any dilution of any flour of known absorption. On a 15% moisture basis the starch used for these investigations had an absorption of 53%.

Experimental

A composite of many southwestern flours was chosen for this study because of its representative character, and the samples of Chiefkan and Cheyenne selection were chosen because of their divergent characteristics. Curves were run on these flours at their normal protein levels and on mixtures of these flours with wheat starch added in amounts calculated to reduce the protein content in decrements of one percent. These curves are shown in Figure 1. The curves are similar to those obtained by Larmour, Working, and Ofelt (1939) on a series of normal flours at different protein levels, and within the normal protein ranges could easily be substituted for a natural series. These curves do not show the decrease in time to minimum mobility with increasing dilution with starch, as was reported by Markley (1938); on the contrary, the curves show an increase to minimum mobility with increasing dilution. This difference in results is probably due to the difference in the absorptions used in the two investigations; in this investigation the predetermined baking absorption was used, while in Markley's investigation the absorption was so adjusted as to give a minimum mobility of 550 Brabender units; that is, the absorption was adjusted to make all curves the same height. Merritt and Bailey (1939) found that this absorption, which gives a minimum mobility of 550 Brabender units, does not correspond to baking



that of the normal flour at the original protein level. The following curves are at dilutions calculated to represent 1% decrements in protein content. The numerical values represent the protein level of the flours and of the flour-starch mixtures.

absorption. It would require considerable readjustment in absorption to bring all the curves in each series shown in Figure 1 to the same height.

That adjusting absorptions to bring all curves to a common height would give an entirely different series of curves from those shown in Figure 1 is shown in Figure 2. This series of curves was run on a 14.8% protein hard winter wheat flour at the naturally occurring protein level and diluted with starch to 9%, 7%, and 6% protein levels. The upper row of curves is for the original flour and for the different dilutions of the flour, each given normal baking absorption, while the curves immediately below are for the same dilutions given absorptions which would bring all curves to a 6-cm. height. It was impossible to bring the curves for mixtures with less than about 6% protein to this particular height, since there is of course a lower limit to the amount of water that may be used to produce a dough. The wide fluctuations in the 6% protein curve are indicative of the dryness of the dough. These curves, of course, do not resemble curves that would be obtained with the Brabender farinograph, but the same tendencies would be evident regardless of the particular shape of the curve.

An observation of interest was that the flour-starch mixtures that were given normal baking absorptions seemed to retain true flour-dough character down to and including those at a 4% protein level. At a level of 3% protein or less the mixtures had starch characteristics; they would not "pick up" in the mixer and did not form the smooth continuous dough characteristic of wheat flours. It should be noted (Fig. 1) that there is an indication of change in the type of curve in each series between the 5% and 6% protein levels.

Curves were run on the original flours at the naturally occurring protein levels in each of the variety-protein series. The curves for these flours are shown in row *A* for each variety in Figure 3. These curves show the same approximate inter- and intra-varietal differences as did curves run by Larmour, Working, and Ofelt (1939) on a similar series of flours from the previous crop year. It should be noted that protein content is not correlated with dough development time; this is not in agreement with Markley, Bailey, and Harrington (1939), again probably as a result of the different methods of determining absorption.

All of the flours in each of the variety-protein series were diluted to a 9% protein level by the addition of wheat starch and curves were run on the resulting flour-starch mixtures. The resulting curves are

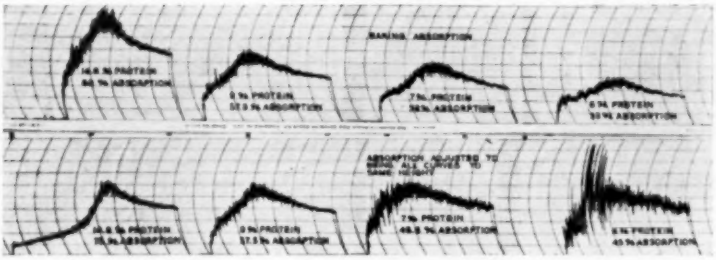


Fig. 2. Curve characteristics of a flour and its dilutions with starch as affected by the use of baking absorption (above) in contrast to absorption adjusted to bring all curves to the same height (below).

shown in row *B* for each variety in Figures 3. The curves for the undiluted flours indicate that the height of the curve is dependent upon the protein content of the flour. Similarly all flours diluted to a constant protein level within a given variety produce curves of a nearly constant height. The heights of curves of the diluted samples from all varieties, with the exception of Chiefkan, Early Blackhull, and Blackhull, were similar. Curves from the diluted samples of Chiefkan and Blackhull were higher at the low protein levels than those from the other varieties and decreased in height with increase in protein content of the original flour used in the flour-starch dilution mixture. The curves for the diluted samples of Early Blackhull were also higher than those from varieties usually considered more desirable but did not exhibit the decline in height shown by the Chiefkan and Blackhull varieties. It should be noted that, while curve height was apparently governed to a large extent by protein content, the curve for the diluted flour retained much of the character or type of the curve obtained from the original flour, and that the curve of the diluted flour varies with the naturally occurring protein level. Sandstedt and

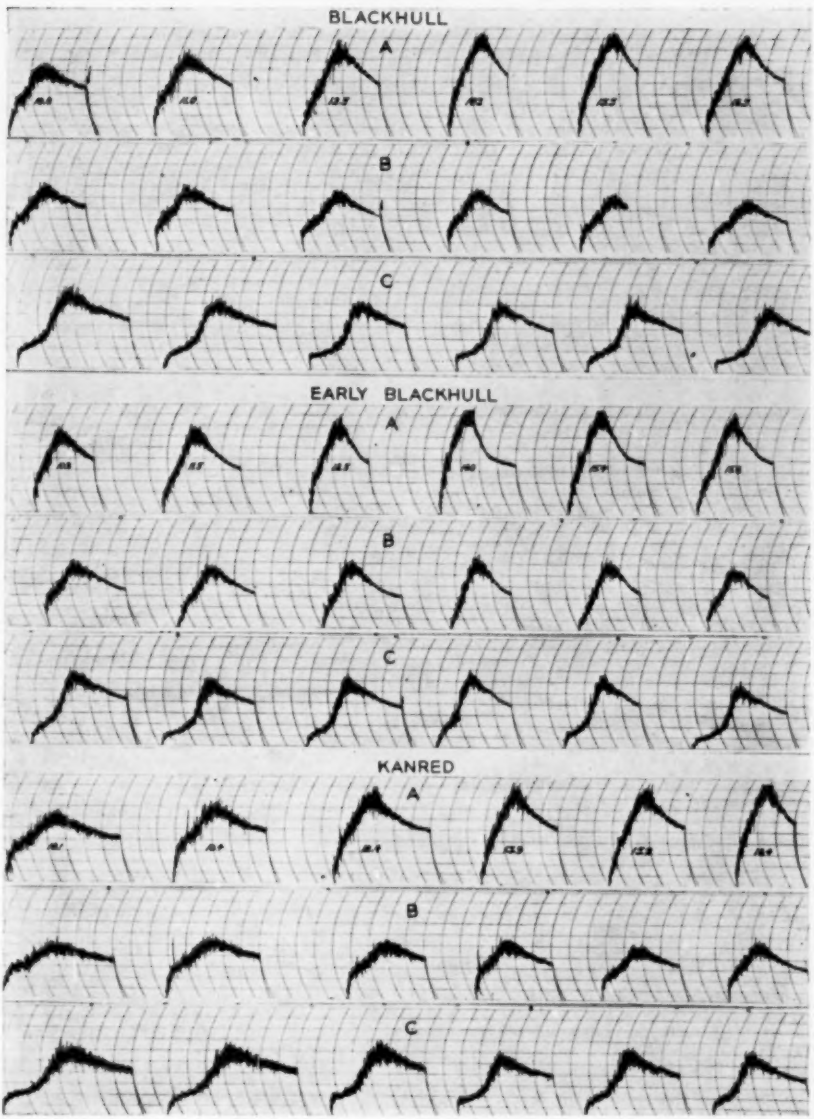
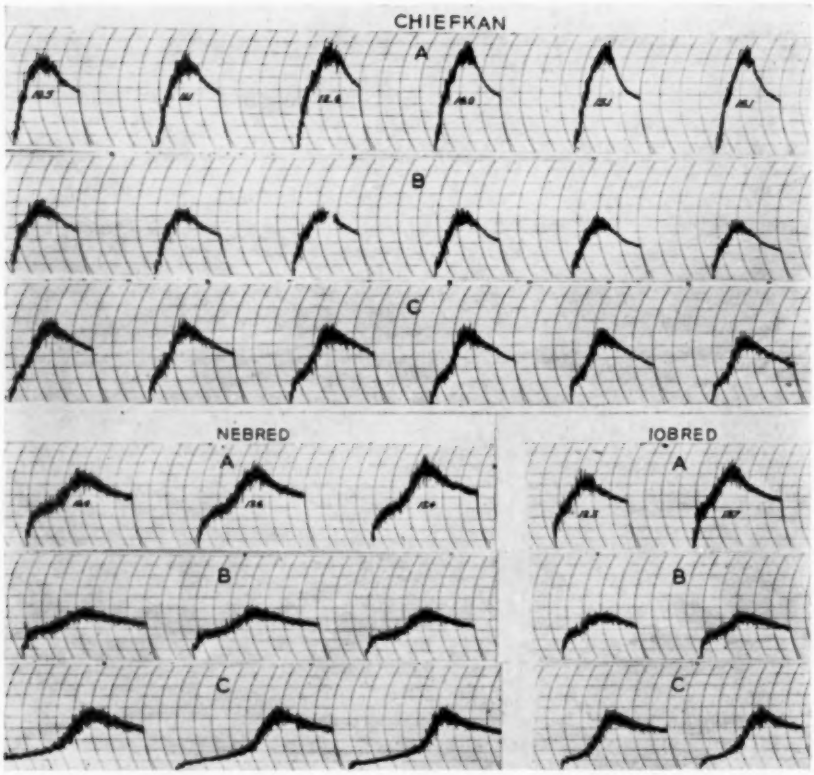


Fig. 3. Recording-micro-mixer curves on flours milled from different varieties of wheat. Each variety is represented at different naturally occurring protein levels and by flour-starch mixtures made from these flours. Curves in series *A* are on the original flours at their indicated protein levels. All curves in series *B* are on flour-starch mixtures obtained by diluting the flour directly above it (in series *A*) to a 9% protein level by addition of wheat starch. Curves in series *C* correspond to those directly above in series *B*, with the exception that all baking ingredients were added.



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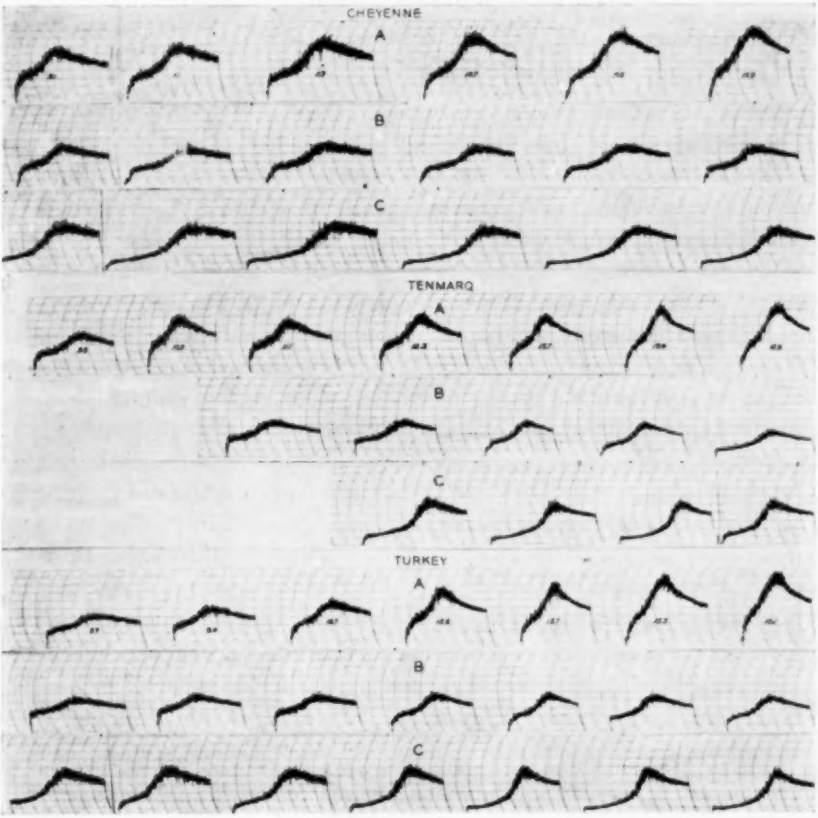

Fig. 3.—*Concluded*

Ofelt (1940) have indicated that baking quality of diluted flours varies in a similar manner.

As a matter of interest, curves were run on the flour-starch dilution mixtures with the addition of baking ingredients. These included sugar 6%, salt 1.75%, shortening 3%, yeast 2%, and dry milk solids 6%. The resulting curves are shown in row *C* for each variety in Figure 3. The same comments with regard to the height and character apply to these curves as to the curves run on the flour-starch dilution mixtures with the simple addition of water.

Summary

Three flours of widely divergent type were used in conjunction with wheat starch to produce flour-starch mixtures with protein levels reduced in decrements of one percent. Recording-mixer curves were obtained from these mixtures at predetermined baking absorptions. The curves were similar to curves obtained from flours with the same

naturally occurring protein content; they showed the same tendency to decrease in height and flatten out with decrease in protein. These curves did not show a decrease in time to minimum mobility with increasing dilution.

Recording-mixer curves were obtained on flours from nine varieties of wheat, each variety being represented by samples at different protein levels. Each of these flours was reduced to a 9% protein level by dilution with wheat starch.

Recording-mixer curves were obtained on these flour-starch mixtures with the addition of water and also with the addition of the baking ingredients normally used in commercial practice. The curves, on both the natural and the diluted flours, show that the height of the curve is largely determined by the protein content and that the time to minimum mobility is not correlated with protein content, *i.e.*, that mixing time is not dependent on protein content but is largely a varietal characteristic. The curve for each of the diluted flours retained much of the character of the original flour. The series of curves for Chiefkan, Blackhull, and Early Blackhull indicate that the protein content is not the only factor determining curve height.

Attention is drawn to the differences in interpretation of curves, which is occasioned by so adjusting absorption as to bring all curves to the same height in contrast to using baking absorption.

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THE LIPIDS OF CORN STARCH[1]

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(Received for publication December 13, 1940)

The lipids of corn starch that cannot be removed by the usual fat solvents make up about one-half of one percent of the commercial starch. This material is liberated upon acid hydrolysis of the starch and appears in the hydrolysate as an insoluble residue. Schoch (1938) has reported that the fatty acids from cereal starches can be removed by Soxhlet extraction with water-miscible fat solvents such as methanol, the cellosolves, or 80% dioxane in water.

Taylor and Nelson (1920) were the first to make a study of the lipids associated with starch. They found that, on acid hydrolysis of purified corn starch, fatty acids were liberated amounting to 0.61% of the dry weight of the starch. They reported that palmitic acid was the principal constituent, and that an unsaturated substance of unknown structure was present. They believed that the palmitic acid was attached only indirectly to the carbohydrate, but directly to the unsaturated component present. Taylor and Lehrman (1926) found the fatty acids obtained from corn starch to be composed of 40% oleic, 36% linolic, and 24% palmitic. Taylor and Iddles (1926) found that only the alpha portion of corn starch contained fatty acids. Rask and Phelps (1925) extracted corn starch with a mixture of ethyl alcohol, concentrated ammonia, and water, and obtained 0.54% "lipoid material" without hydrolyzing or gelatinizing the starch. Taylor and Werntz (1927) found that the material extracted by this reagent was a mixture of fatty acids and extraneous material and that only 20% to 25% of the fatty acids present in corn starch were removed by this treatment. Taylor and Sherman (1933) found that the fatty acids were liberated preferentially during acid, diastatic, or alkaline hydrolyses of corn starch and that the unsaturated acids were most easily removed. Schoch (1938) suggested that since free fatty acids can be extracted from cereal starches with water-miscible fat-solvents, it would appear that the fatty acids were distributed throughout the starch granule as an extraneous impurity and not in chemical combination with the starch.

The present investigation was undertaken for the purpose of making a more detailed study of the lipids from corn starch than has been

[1] Paper No. 1865, Scientific Journal Series, Minnesota Agricultural Experiment Station. Condensed from part of a thesis presented by James W. Evans to the faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June, 1940.

made before. This paper reports the results of a study on the extraction of corn starch with methanol, and of the analysis of the lipids in this extract. The analyses of the lipid material removed from corn starch by acid and by diastatic hydrolysis are also given. The fatty acids present in the starch have been separated and identified.

Fatty Material in Starch Obtained from Corn Kernels Where the Embryos Were Removed before Milling

In the commercial milling process a portion of the starch comes into intimate contact with the embryos when the steeped corn is put through disintegrating mills to rupture the kernel, and when this macerated corn is put into tanks where the germ floats off.

In order to be certain that starch did not adsorb or pick up its fatty acid content during this process, the germs from 500 g. of Reid's Yellow Dent corn were removed from the grains before processing. The endosperms were then steeped at 50°C. for 24 hours in water containing 0.25% sulfur dioxide. The steep water was removed and the process repeated. The steeped endosperms were ground in a Buhr mill, sufficient water being added to make a final "slop" with about 60% water. The ground material was screened and the material that passed through the screen was bolted on No. 17 bolting silk (17 XX; 163 meshes per inch). Starch was separated from gluten by suspending the mixture in water, centrifuging, and then scraping the gluten off the settled material with a spatula. The process was repeated four times and on the third centrifuging no gluten was observed on the surface of the starch cake. This purified starch was air-dried for three days and then dried in a vacuum oven at 100°C. for five hours.

The lipid content of the dry starch was determined by the acid hydrolysis procedure of Taylor and Nelson (1920), which is described below under the heading "Analysis of lipids liberated by acid hydrolysis." The final ether extract of the residue from hydrolysis was dried in vacuum at 50°C. to constant weight.

The fatty material thus liberated from the starch was found to be 0.64% on dry substance basis (as compared with 0.61% found by Taylor and Nelson for purified corn starch).

This shows that the fatty material ordinarily present in corn starch does not get on or into the starch during the milling process.

Preparation and Analysis of Factory Starch

The commercial corn starch used in this work was supplied by the Union Starch and Refining Company of Granite City, Illinois. It was produced by the wet milling process and was taken from the fourth American filter. After being air-dried in the laboratory, the moisture

content of this starch was found to be 11.5%. Analysis of the starch, calculated on a dry-substance basis, gave the following results: protein, 0.43%; ash, 0.10%; fat (by hydrolysis), 0.64%; materials extractable by ether, 0.062%; and phosphorus, 0.016%.

Extraction of Starch

While ether extraction of the products of acid hydrolysis of starch yields about 0.62% of the dry weight of the starch as ether-soluble substances, it is not possible to remove more than a small fraction of these lipids by ether extraction of the original dry starch. Analysis of the lipids obtained after acid hydrolysis must necessarily fail to give much information as to the actual state of combination characteristic of the lipids in the original starch. Whether or not the fatty acids, which make up the larger percentage of the lipids obtained after acid hydrolysis, are present in the starch as such, or in combination with glycerol (or with the starch itself), is a question of much importance. Water-soluble fat solvents have been shown to remove a fairly high fraction of the lipids present. If a complete, or nearly complete, extraction of the lipids could be obtained by using such solvents, the question as to the state of the fatty acids in the starch, before hydrolysis, could be answered by analysis of such extracts.

For the following extraction experiments, factory starch that had been dried in vacuum at 100°C. for 12 hours was used.

1. Soxhlet extraction with methanol as solvent.—Samples of dried starch (50 g. each) were placed in 42 × 120 mm. paper extraction thimbles, and extracted with absolute methanol (A. R. grade) in Soxhlet extractors for 24, 36, and 48 hours, respectively. At the end of the extraction period the methanol was distilled off and the flasks were dried in an oven at 50°C. to a constant weight (methanol extract). Ether was then added to the flasks, refluxed for 10 minutes, cooled, filtered into a tared flask, the ether removed on a steam bath and the flasks dried to constant weight (methanol extract soluble in ether). To obtain the amount of the methanol extract that was soluble in water, the original dried extract was mixed with water and allowed to stand for 24 hours with occasional stirring, then filtered and the filtrate evaporated to dryness. Kjeldahl nitrogen was determined on the methanol extract, the water-soluble portion, and the portion that was insoluble in water and ether but soluble in methanol. The fatty material was determined in the extracted starch by liberating with acid hydrolysis as described below. Results of these analyses are given in Table I.

It is seen that not all of the fatty material can be removed from the starch by the extraction method described above. Nor is all of the

material extracted by methanol of a lipid nature. This is as would be expected, since the starch contained 0.43% protein. The results indicate that some protein is peptized by the methanol, and also that some water-soluble material which is not protein is removed. (This material shows no reducing properties when tested with a small amount of Benedict's solution.)

TABLE I

ANALYSES (AS PERCENTAGES OF DRY WEIGHT OF THE STARCH) OF THE EXTRACT AND OF THE EXTRACTED STARCH OBTAINED THROUGH METHANOL EXTRACTION OF DRY STARCH IN A SOXHLET EXTRACTOR

	Time of extraction (hours)		
	24	36	48
	%	%	%
Methanol extract	0.68	0.78	0.79
Methanol extract soluble in ether	0.40	0.46	0.48
Methanol extract soluble in water	0.14	0.14	0.15
Methanol extract insoluble in ether and water	0.10	0.15	0.16
Protein in methanol extract	0.06	0.11	0.12
Protein in methanol extract soluble in water	0.02	0.02	0.02
Protein in methanol extract insoluble in ether and water	0.05	0.08	0.10
Fatty material liberated from extracted starch by acid hydrolysis	0.23	0.18	0.17

2. Soxhlet extraction with methanol as solvent on starch that had been ground in rod mill.—Since all of the fatty material was not removed by the above procedure, it was thought that, by grinding the starch in a rod mill to break the granules and then extracting with methanol, more of the lipids might be removed.

Samples of 200 g. of starch were ground for 75 and 150 hours in a rod mill that was designed by Stamberg and Bailey (1939). The mill consisted of a one-gallon ball-mill jar with 30 steel rods 13 cm. long and 2 cm. in diameter. The jar rotated at the rate of 13.0 rpm. After grinding, the starch was extracted for 48 hours as previously described. The results are given below:

Time in mill (hours)	75	150
Methanol extract (%)	0.77	0.79

The starch that had been ground for 75 hours and extracted with methanol for 48 hours was extracted for an additional 104 hours with only a 0.01% increase in extractable material.

No more material could be extracted from the ground starch than from the original. It appears, therefore, that the complete removal of the fatty substances with methanol is not dependent upon the disruption of the starch granule. In view of this finding it would seem

unlikely that the fat is distributed through the starch by simple occlusion.

3. Soxhlet extraction with other solvents.—Neither ethanol nor a 70/30 mixture of methanol and ether gave better results than did methanol alone.

4. Methanol extraction in a Stokes extractor.—In the Soxhlet extractor, the extraction is effected by cold solvent. Extraction with hot solvent was tried, employing an extractor described by Stokes (1914). The sample to be extracted was placed in a thimble, covered by a plug of fat-free cotton, and supported in the flask by means of a wire passing through the cork connecting the flask with the condenser. The thimble containing the sample was allowed to remain in contact with the boiling methanol for 12 hours and was then pulled up out of the solvent (by means of the wire) and extracted by the boiling vapors for an additional 24 hours.

TABLE II

ANALYSES (AS PERCENTAGES OF DRY WEIGHT OF STARCH) OF EXTRACT AND OF THE EXTRACTED STARCH OBTAINED AFTER HOT METHANOL EXTRACTION OF DRY STARCH IN A STOKES EXTRACTOR

	Sample (grams)			
	18.0	20.0	73.7	70.0
	%	%	%	%
Methanol extract	1.06	1.00	1.03	1.00
Methanol extract soluble in ether	0.66	0.62	0.65	0.62
Fatty material liberated from extracted starch by acid hydrolysis	0.04	0.04	0.03	0.04

The weights of the methanol extract and of the fraction of the methanol extract which was soluble in ether were determined as described above. Fatty materials remaining in the extracted starch were freed by acid hydrolysis and determined. Results are those shown in Table II.

The same procedure was carried out, using starch containing 10% moisture in place of dry starch. After the extraction was completed, sufficient ether was added to the extract to make about a 70/30 methanol-ether mixture. Anhydrous sodium sulfate was added and the mixture was shaken and allowed to stand for 24 hours. Then the sodium sulfate was filtered from the extract, broken up in a mortar, and washed three times with dry ether, the washings being added to the extract. The results are shown in Table III.

These results indicate that practically all (95%) of the lipid material can be removed from corn starch by extracting with hot methanol and that it is not necessary to use dry starch in the process. The

TABLE III

ANALYSES (AS PERCENTAGES OF DRY WEIGHT OF STARCH) OF EXTRACT AND OF THE EXTRACTED STARCH OBTAINED AFTER HOT METHANOL EXTRACTION OF STARCH CONTAINING 10% MOISTURE IN A STOKES EXTRACTOR

	Sample (grams)		
	70.0	70.0	70.0
	%	%	%
Methanol extract	1.00	1.05	1.08
Methanol extract soluble in ether	0.62	0.64	0.62
Fatty material liberated from extracted starch by acid hydrolysis	0.04	0.03	0.06

extracted starch retained its characteristic granular structure and its ability to form a paste with hot water, as was also observed by Schoch (1938).

Analysis of Lipids Obtained from Starch by Methanol Extraction, by Acid Hydrolysis, and by Diastatic Hydrolysis

1. Preparation of lipid samples: (*a*) *Extracted by methanol.*—A 2,100-g. sample of factory starch (1,890 g. dry substance) was extracted with methanol by Stokes' method. Sufficient ether was added to the extract to make a 70/30 methanol ether mixture and this was dried two days with freshly ignited sodium sulfate. After filtering, the solvents were distilled off, under diminished pressure, in a current of nitrogen, the temperature never exceeding 40°C. The residue was extracted with anhydrous ether, the solution filtered, and made to 1,000 ml. The fat content of this solution, as determined on several 25-ml. aliquots, was equivalent to 0.61% of the starch (d.s. basis).

For all the lipid determinations an aliquot of the above solution was used, the concentration being checked each time by evaporating a larger or an equal aliquot in a tared flask. The solvent was distilled off under diminished pressure in a current of nitrogen.

(*b*) *Liberated by acid hydrolysis.*—A 15,000-g. sample of factory starch (13,500 g. dry substance) was hydrolyzed with hydrochloric acid in essentially the same manner as described by Taylor and Nelson (1920). Batches of the starch were suspended in four times their volume of water. This suspension was poured into approximately three times its volume of boiling hydrochloric acid solution (made by adding 1 volume of concentrated hydrochloric to 2½ volumes of water). Boiling was continued until a test sample gave no color with iodine. The mixture was then cooled to 20°C., filtered, and the residue was washed with cold water until the washings were free of chlorides.

After the residue and filter paper had been dried for 4 hours at 50°C. in vacuum oven, they were placed in a Soxhlet extractor and extracted with ethyl ether for 28 hours. This ether extract was made up to volume with ether and aliquots of this solution were used for the various lipid determinations. The hydrolysis was carried out in an atmosphere of nitrogen and a stream of nitrogen was passed through the vacuum oven during drying of the residues in order to prevent oxidation of the unsaturated fatty acids.

(*c*) *Liberated by diastatic hydrolysis.*—To obtain the lipids for this work, 1,110 g. of starch (1,000 g. dry substance) were hydrolyzed with malt extract. The hydrolysis was carried out on ten "111-gram" samples as follows:

The enzyme was obtained by stirring 100 g. of ground "malted" barley in 600 ml. of water for one hour, then centrifuging and filtering. Two ml. of this extract was used for each gram of starch.

A 111-g. sample of starch was suspended in 200 ml. of water, and added to 1,000 ml. of boiling water, with constant stirring. The mixture was boiled for 30 minutes, and then cooled to 50°C. To this, 220 ml. of malt extract was added and the mixture digested until there was no color produced when tested with iodine (usually 24 hours). During the digestion period, a stream of nitrogen was slowly bubbled through the suspension to prevent oxidation. At the end of this period, the material was cooled to about 20°C., filtered, and the residue washed until the filtrate showed no reducing power when tested with Fehling's solution. The residue was dried in a stream of nitrogen in a vacuum oven at 50°C. for 6 to 8 hours and then extracted with ether in a Soxhlet extractor for 24 hours.

The ethereal extracts were concentrated to about 700 ml., dried with anhydrous sodium sulfate, filtered, and made up to 1000 ml. with dry ether. Aliquots of this solution were used in all determinations.

2. Lipid analysis.—Iodine value (Wijs), acid value, saponification value, total fatty acids, and ester value were determined as described by Jamieson (1932). The thiocyanogen value was determined by the Kaufmann method as described by McKinney (1938). Saturated and unsaturated fatty acids were separated by the Twitchell (1921) procedure. The iodine numbers were determined also by the Rosenmund-Kuhnhenn (1923) method which uses pyridine sulfate dibromide as the halogenating agent. Unsaponifiable matter was determined by the Allen and Thomson method as described by Lewkowitsch (1909), and the phosphatides by Boyd's (1931) modification of Bloor's (1929) oxidation method. Saponification was carried out in an atmosphere of nitrogen in order to prevent oxidation of the unsaturated fatty acids.

3. Calculation of the unsaturated fatty acids.—The amounts of the various unsaturated fatty acids present in the total fatty acids were calculated according to the following equations of Kaufmann and Keller (1929).

$$G + Ln + L + O = 100$$
$$273.7\ Ln + 181.1\ L + 89.9\ O = 100\ (I.V.)$$
$$182.5\ Ln + 90.5\ L + 89.9\ O = 100\ (T.V.)$$

G = % saturated acids (from Twitchell separation)
Ln = % linolenic acid
L = % linolic acid
O = % oleic acid
I.V. = iodine value of total fatty acids (Wijs)
T.V. = thiocyanogen value of total fatty acids

The above equations can be simplified as follows:

Let:

$$\text{Unsaturated fatty acids (U.A.)} = 100 - G$$

Then:

$$O = 0.994\ U.A. + 1.113\ T.V. - 1.104\ I.V.$$
$$L = 0.984\ U.A. + 1.112\ I.V. - 2.209\ T.V.$$
$$Ln = 1.096\ T.V. - 0.0076\ I.V. - 0.978\ U.A.$$

When oleic, linolic, and linolenic acids are present, the validity of the method for calculating their relative amounts depends upon the validity of the assumption that the theoretical iodine and thiocyanogen values are correct. Kass, Lundberg, and Burr (1940) have recently shown that the iodine numbers (Wijs) of pure linolic and linolenic acids agree with the theoretical values, but that the thiocyanogen values of these acids, determined according to the officially recommended methods, are 96.3 and 171 respectively. These values will vary, also, with the conditions of the determination. They suggest that empirical values be substituted for the theoretical constants in the equations for the calculation of the amounts of the unsaturated fatty acids. However, the equations given above have been employed in arriving at the distribution of unsaturated acids reported in this paper.

In most cases where a mixture of saturated acids, oleic, linolic, and linolenic acids are present, the use of the thiocyanogen procedure gives a valuable index as to the types and amounts of the fatty acids present. Some workers have found difficulty in using the method, but if Kaufmann's procedure is followed, Griffiths and Hilditch (1934) and Kimura (1929) have shown that it gives good results.

The analyses of the lipids obtained from the starch by the three methods described, and the calculated distributions of the fatty acids in the total fatty acids of the lipids, are shown in Table IV.

The methanol extract gave a positive test for glycerol, but the amount was not sufficient for a quantitative determination.

It can be seen, from the above results, that the mixtures of fatty acids obtained after saponification of the lipids extracted from starch

by methanol and those obtained after acid hydrolysis of the starch are very similar in composition. Therefore, in a study of the fatty acids of corn starch, those obtained by either method could be employed with equal accuracy. Diastatic hydrolysis does not appear to liberate all of the fatty acids from starch. The unsaturated acids are liberated more easily than the saturated ones.

TABLE IV

ANALYSES OF THE LIPIDS OBTAINED FROM COMMERCIAL CORN STARCH BY THREE METHODS OF PREPARATION

	Extracted with methanol	Liberated by acid hydrol-ysis	Liberated by diastatic hydrolysis
Methanol extract (*% of starch*)	1.10	—	—
Lipids (*% starch*)	0.61	0.64	0.55
Acid value	164.4	195.1	177.0
Saponification value	186.6	—	205.6
Ester value	22.2	—	28.6
Iodine value:			
Rosenmund-Kuhnhenn	88.5	85.9	—
Wijs	93.5	90.5	93.5
Thiocyanogen value	58.2	57.8	65.3
Unsaponifiable matter (*% of lipid material*)	8.0	5.2	8.0
Phosphatids (*% of lipid material*)	0.7		0.6
Total fatty acids (*% of lipid material*)	91.0	93.8	90.5
Acid value	203.0	202.8	202.5
Mean molecular weight	276.4	276.5	277.0
Iodine value:			
Rosenmund-Kuhnhenn	85.9	86.4	—
Wijs	93.2	93.6	103.2
Thiocyanogen value	63.6	64.2	70.7
Saturated fatty acids (Twitchell) (*% total f.a.*)	30.5	30.0	23.0
Iodine value	0.8	0.9	0.9
Mean molecular weight	268.2	269.0	267.9
Unsaturated fatty acids (by diff.) (*% total f.a.*)	69.5	70.0	77.0
Mean molecular weight	280.1	280.4	280.8
Iodine value:			
Rosenmund-Kuhnhenn	123.0	122.2	—
Wijs	134.0	133.0	131.6
Oleic acid (*% of total fatty acids*)	37.0	37.7	41.3
Linolic acid (*% of total fatty acids*)	31.5	31.1	34.3
Linolenic acid (*% of total fatty acids*)	1.0	1.2	1.4

The results of the present work show that the major portion of the fatty acids are removed in the free form by both methanol extraction and diastatic hydrolysis. The methanol extraction would not be expected to hydrolyze the fatty acid esters, for, if it did, the acids in the extract would have been in the form of methyl esters since the condition that favors hydrolysis would also favor the formation of methyl esters.

In order to ascertain if the malt extract contained lipases that would split fatty acids from a glyceride, olive oil was subjected to the action of the extract. To 4 ml. of olive oil, 60 ml. of malt extract was added and the mixture was digested for 24 hours at 50°C. At the end of this period the material was cooled and filtered. The filter and residue were dried in a vacuum oven at 50°C. for 8 hours and then extracted with ether for 4 hours. Before this treatment the olive oil had an acid value of 1.84 and after treatment 1.87. This shows that there is very little, if any, hydrolysis of the glycerids by malt extract.

In view of the above it may be concluded that most of the fatty acids, present in starch, are in the form of free fatty acids.

Analysis of the Lipids Removed from Starch at Various Degrees of Extraction with Methanol and the Analysis of the Extracted Starch for Fatty Acids and Phosphorus

1. Lipid analysis.—In the course of the study of methods for extracting lipids from starch, it was found that the iodine value for the extracted fat was highest when the smallest amount of material had been removed. Accordingly, it seemed of interest to analyze the fatty material liberated, and also that remaining in the starch, at different degrees of extraction.

Starch was extracted with methanol in the following manner: A 2,000-g. sample of starch (11% moisture) was suspended in 3 liters of methanol and stirred for 4 hours and then allowed to stand overnight. One liter of the solvent was withdrawn from above the starch cake, filtered, and a portion of the methanol distilled off. A liter of methanol was added to the starch cake and the mixture was stirred for 2 hours. It was allowed to stand for an additional 2 hours, then a liter of solvent was withdrawn, filtered, and distilled. This process was continued until 12 liters of solvent had been removed. The starch was then collected on a Büchner funnel and dried, first in air and then in an oven at 60°C. This procedure removed 57% of the total lipids in the starch.

To obtain a higher removal of fatty material, a 980-g. sample of starch was extracted in a Stokes' extractor for 12 hours. This extracted 81% of the total lipids.

The lipids were obtained from the methanol extracts by adding sufficient ether to make the mixture about 30% ether. This was dried over anhydrous sodium sulfate for 2 days, and after filtering the solvents were distilled off under reduced pressure in a stream of nitrogen, the temperature never exceeding 40°C. This residue was extracted with ether, the solution filtered, and made up to volume. The methods of analysis were the same as those described previously.

Lipids remaining in the extracted starches were obtained by acid hydrolysis of the material followed by collection, washing, drying, and extraction of the flocculent substance formed by hydrolysis. This procedure has also been described. Results of this work are given in Table V.

TABLE V

ANALYSIS OF THE LIPIDS OBTAINED FROM AND REMAINING IN COMMERCIAL CORN STARCH AT VARYING DEGREES OF COMPLETENESS OF EXTRACTION WITH HOT METHANOL

	Lipids removed by methanol extraction			Lipids remaining in starch—liberated by acid hydrolysis		
Total fat extracted Total fat remaining	57% —	81% —	95% —	— 19%	— 43%	— 100%
Lipids (% *of starch*)	0.36	0.52	0.61	0.11	0.30	0.64
Acid value	165.1	177.0	164.4		195.6	195.1
Iodine value:						
Rosenmund-Kuhnhenn	105.5	92.7	88.5		49.9	85.9
Wijs	111.2	104.0	93.5		53.0	90.5
Thiocyanogen value	74.7	64.8	58.2		46.2	57.8
Unsaponifiable matter (% *of lipid material*)	10.0	9.0	8.0	4.0	3.0	5.2
Total fatty acids (% *of lipid material*)	89.1	90.4	91.0	95.1	96.1	93.8
Acid value	199.5	201.5	203.0	205.8	204.2	202.8
Mean molecular weight	280.7	278.5	276.4	272.8	274.5	276.5
Iodine value:						
Rosenmund-Kuhnhenn	109.7	96.0	85.9	43.5	57.6	86.4
Wijs	115.8	104.5	93.2	47.2	61.4	93.6
Thiocyanogen value	76.4	72.0	63.6	31.0	45.2	64.2
Saturated fatty acids (Twitchell) (% *total f.a.*)	16.2	21.5	30.5	66.3	50.0	30.0
Iodine value	0.9		0.8	0.8		0.9
Mean molecular weight	267.7	267.5	268.2	269.3	269.0	269.0
Unsaturated fatty acids (by diff.) (% *total f.a.*)	83.8	78.5	69.5	33.7	50.0	70.0
Mean molecular weight	280.8	281.3	280.1	278.1	282.0	280.4
Iodine value:						
Rosenmund-Kuhnhenn	126.8	122.4	123.0	128.5	115.0	122.2
Wijs	137.7	132.0	134.0	140.0	123.4	133.0
Oleic acid (% *of total fatty acids*)	40.5	42.8	37.0	15.9	32.2	37.7
Linolic acid (% *of total fatty acids*)	42.4	34.4	31.5	17.1	17.6	31.1
Linolenic acid (% *of total fatty acids*)	0.9	1.3	1.0	0.7	0.2	1.2

The results in Table V show that the unsaturated fatty acids are more easily removed by methanol extraction than are the saturated acids. This can be accounted for in the light of the following experiment in which it was found that methanol-extracted corn starch preferentially adsorbed palmitic acid from a methanol solution of palmitic and oleic acids.

A solution containing 10.7 g. oleic acid and 8.7 g. palmitic acid in sufficient methanol to make 100 ml. was divided into two equal

portions. One portion was poured into a flask containing 10 g. of fat-free starch (extracted with methanol) and the other portion was filtered through filter paper. The flask containing fatty acids and starch was shaken for 20 minutes and then filtered. The solids and iodine value were determined on 5-ml. aliquots of the filtrates. It was found that whereas the fatty acid mixture in the control filtrate showed an iodine value of 50.1 and a total solid content of 0.9694 g. in a 5-ml. aliquot ,the fatty acid mixture obtained in the filtrate from the starch showed an iodine value of 59.3 and a total solid content of 0.9420 g. in a 5-ml. aliquot.

As the fatty acids in the filtrate from the starch were more unsaturated than the acids in the control, it can be concluded that more palmitic than oleic acid had been adsorbed by the starch. The saturated acids are held more firmly by starch than are the unsaturated acids and hence are more difficult to remove.

The above data also show that, in a study of the fatty acids removed from a starch by methanol extraction, care must be taken to remove all of the lipids in order to obtain the correct acid composition.

2. Phosphorus content of extracted starch.—Taylor (1928) has pointed out an interesting correlation between the phosphorus content of a few common starches and their associated fatty acid content. He observed that the starches which were low in fatty acid were correspondingly high in phosphorus content. It was considered of interest to follow the change in phosphorus content of the starch during the fractional removal of the lipids by methanol extraction. The phosphorus content of the starches was determined by the method of Morris, Nelson, and Palmer (1931).

It was found that, whereas the original starch contained 0.016% phosphorus (dry substance basis), after 57% of the lipids had been removed by methanol extraction, the phosphorus content of the starch was 0.015%. After 81% had been removed, the phosphorus content of the starch was 0.010% and, after 95% had been removed (highest fraction of total lipids removable by methanol), the phosphorus content was still 0.010%. Phospholipids in the methanol extract (95%, see Table IV) amounted to only 0.7% of the total weight of the lipids present. This corresponds to only 0.00016% phosphorus, as phospholipid, in the starch. Methanol extraction removed phosphorus, however, to the extent of 0.006% of the weight of the starch. It is apparent, then, that the major part of the phosphorus removed by the methanol extraction of the starch is not identifiable as phospholipid. No attempt was made to identify this major portion of the extractable phosphorus.

Identification of the Unsaturated Fatty Acids

1. Bromination, separation and identification of the bromides.—A quantity of unsaturated fatty acids (separated by the Twitchell procedure) was dissolved in absolute ethyl ether and brominated according to the procedure of Bailey and Johnson (1918), using bromine dissolved in glacial acetic and a reaction temperature of 2°–3°C. Duplicate samples were run through the bromination and procedure for the separation of the bromides.

After addition of sufficient bromine (so that a deep red color remained in the solution) the mixture was allowed to stand in a refrigerator at about 0°C. for from 16 to 24 hours, then filtered through a sintered glass crucible (10G3) and washed with three 10-ml. portions of ice-cold dry ethyl ether. (The absolute ether was prepared by washing c.p. ether three times with one quarter of its volume of 3% potassium permanganate. It was then washed twice with a saturated solution of calcium chloride and allowed to stand over dry calcium chloride, then filtered and stored over sodium.) The residue that remained on the filter, and which was found to be completely soluble in benzene, is designated, in accordance with the nomenclature of Kaufmann and Keller (1929), as α-hexabromide and corresponds, therefore, to a part of the linolenic acid present in the mixture of unsaturated acid. (The β- or soluble hexabromide is neglected in the subsequent analysis of the bromides.)

The ether was evaporated from the filtrate under diminished pressure, the last portion being removed by heating on a steam bath. The residue was dissolved in a small volume of petroleum ether (b.p. 30°–60°), then refluxed, cooled, and put in a refrigerator at 5°C. for 16 hours. The precipitate was filtered off on a sintered glass crucible, washed with cold petroleum ether, and dried at 95°C. The precipitate is designated as α-tetrabromide and corresponds to part of the linolic acid present in the original mixture of unsaturated acids. The precipitates from the duplicate determinations were combined, dissolved in petroleum ether, decolorized with "Norit," and recrystallized. White needles were obtained which melted from 113° to 115°C. (Dean, 1938, gives 114°C. as the melting point for linolic α-tetrabromide.) Bromine determinations, made on duplicate samples of this precipitate by the method described by Tolman (1909), gave weights of silver bromide that corresponded to an average of 53.6% bromine in the compound. The theoretical value for linolic tetrabromide in 53.33%.

The petroleum ether filtrate from the linolic α-tetrabromide was evaporated to dryness and taken up with dry ethyl ether. The ether solution was washed with sodium sulfite to remove the excess bromine,

and then evaporated to dryness. The bromine content of this residue was determined by the same procedure as was used previously. The silver bromide produced corresponded to an average of 42.05% bromine in the residues from the duplicate separation procedures. The theoretical bromine content of oleic dibromide is 36.18%. The high value obtained is due to the presence of some linolic tetrabromide (the so-called β-tetrabromide).

On the assumption that no hexabromides and that only oleic dibromide and linolic tetrabromide are present in the residue, its relative composition was calculated in the following manner:

$$\begin{aligned} X &= \text{oleic dibromide (\% of residue)} \\ 100 - X &= \text{linolic tetrabromide (\% of residue)} \\ 36.18X + 53.33(100 - X) &= 42.05 \\ X &= 65.8\% \text{ oleic dibromide} \\ 100 - X &= 34.2\% \text{ linolic tetrabromide } (\beta) \end{aligned}$$

Results of these separations and calculations are shown in Table VI.

TABLE VI

ANALYSIS OF DUPLICATE SAMPLES OF UNSATURATED FATTY ACIDS BY BROMINATION AND SEPARATION OF BROMIDES

	Samples	
	1	2
	g.	g.
Mixed unsaturated acids	1.600	1.718
α-Hexabromide (insol. in ethyl ether)	0.036	0.036
α-Tetrabromide (insol. in pet. ether)	0.685	0.827
Residue (soluble in pet. ether)	1.980	2.152
Dibromide (calculated, see text)	1.303	1.420
β-Tetrabromide (calculated, see text)	0.677	0.732

In Table VII the values for the composition of the unsaturated acids as determined by the bromination method are compared with those obtained by application of the Kaufmann and Keller equations as already described.

The remainders of the oleic dibromide and linolic β-tetrabromide mixtures were combined. This was debrominated by the method of Rollet (1909). The fatty acids were recovered by saponifying the esters with 4% potassium hydroxide, followed by acidification. The acids were taken up in petroleum ether, washed, and then dried with anhydrous sodium sulfate. The iodine value (Wijs) of the recovered acid was 110.8, whereas the theoretical iodine value for oleic acid is 89.9.

The *p*-phenylphenacyl ester of this material was prepared according to Drake and Bronitsky (1930), and after three recrystallizations from alcohol, the ester had a melting point of 59.5°C. These workers

report a melting point of 60.5°C. for the *p*-phenylphenacyl ester of pure oleic acid.

2. The lithium salt of oleic acid.—Moore (1919) used the lithium salt for the preparation of pure oleic acid. He pointed out that, when a divalent element such as barium is used for the separation of monovalent acids, there is a possibility of double salt formation, as barium oleolinolate, and for this reason he preferred the use of a monovalent element. Neither the barium nor the lithium salts effect a quantitative removal of oleic acid from a mixture of unsaturated fatty acids, but both are useful for the qualitative detection of oleic acid.

TABLE VII

A Comparison of the Relative Composition of the Unsaturated Fatty Acids Present in Starch as Determined by the Bromide Separation Procedure and by the Kaufmann and Keller Technique

	By bromation	By *K* and *K* equation
Oleic acid (% *of total unsat. acids*)	52.0, 52.6 } 52.3	53.2
Linolic acid (% *of total unsat. acids*):		
From α-tetrabromide	20.0, 21.6 } 20.8	—
Brom β-tetrabromide	19.8, 20.0 } 19.9	—
Total	40.7	45.4
Linolenic acid (% *of total unsat. acids*):		
From α-hexabromide	0.84, 0.79 } 0.82	1.4

For the formation of lithium oleate from the unsaturated fatty acids from corn starch, 2.75 g. of the unsaturated acids, separated by the Twitchell method, were dissolved in 15 ml. of absolute ethyl alcohol and heated to boiling under a reflux condenser. Then 0.7 g. of lithium hydroxide dissolved in 15 ml. boiling water was added. (The solution was alkaline to phenolphthalein.) The solution was cooled slowly and placed in the refrigerator for 12 hours. The precipitate was filtered off and washed with 50% alcohol.

The crystalline lithium salt was recrystallized two times from 50% ethyl alcohol and then added to a 10% hydrochloric acid solution to liberate the oleic acid. The fatty acid was extracted with ether, the ether solution washed with water four times, dried with anhydrous sodium sulfate, and made up to 250 ml. The ether was evaporated from a 50-ml. aliquot. The residue weighed 0.0950 g., making 0.475 g. from the sample of 2.75 g. of unsaturated fatty acids. The yield was 17.3%, which is lower than the percentage (52.3) obtained by difference in the bromination procedure or the value (53.2) by Kaufmann and

Keller calculation method, which shows that a quantitative yield was not obtained.

The iodine number of the acid was 87.7 (Wijs), which indicates a rather pure oleic acid.

This result is another proof of the presence of oleic acid in the fatty acids from corn starch.

3. Identification of the saturated fatty acids.—The saturated fatty acids were separated by the Twitchell method from the total fatty acids liberated from corn starch by acid hydrolysis. From 80.0 g. of total fatty acids, 24.0 g. of saturated fatty acids was obtained which had an iodine value of 0.7 and a mean molecular weight of 269 by titration. The acids were esterified to the methyl esters by the procedure described by Jamieson and Baughman (1920).

TABLE VIII

DATA ON THE SEPARATION OF THE METHYL ESTERS OF THE SATURATED ACIDS OF STARCH LIPIDS AND ON THE IDENTIFICATION OF THESE ACIDS

Methyl esters (14.0 g. total)					Acids recovered		Derivatives—M.P. (°C.)	
Fractions	Boiling range	Weight	% of total	M.P.	Saponification value	Mol. wt.	*p*-bromphenacyl ester	*p*-phenylphenacyl ester
	°C.	g.		°C.				
1	145–151	9.66	70.5	30–31	217.7	257.7	85–86	—
2	156–165	3.55	25.9	39	196.7	285.3	90	90.5
3	170	0.49	3.6	55–56	178.8(?)	314.0(?)	89.5	—

Fourteen g. of the mixed methyl esters was distilled at 2 mm. pressure in a micro still equipped with a heated column. Three fractions were obtained, one of which remained in the distillation bulb.

The following boiling ranges were selected because the temperature increased gradually from 145° to 151°C., then moved rapidly to 156°C. From this point to 165° there was another gradual increase and then a rapid increase to 179° where the distillation was stopped. Each fraction was saponified with 4% alcoholic potassium hydroxide, acidified with hydrochloric acid, and the fatty acids obtained in ether. The saponification value was determined for the acid from each fraction and the molecular weight calculated from this.

The *p*-bromphenacyl ester of the acid obtained from each fraction was prepared according to the method of Hann, Reid, and Jamieson (1930) and the *p*-phenylphenacyl ester of the acid obtained in fraction No. 2 was prepared according to the method of Drake and Bronitsky (1930).

The results of these experiments are given in Table VIII. The molecular weights of palmitic, stearic, and arachidic acids are 256.3,

284.3, and 312.3, respectively. Hann, Reid, and Jamieson have reported the melting points of the *p*-bromphenacyl esters of these acids to be 86°C., 90°C., and 89.5°C., respectively. The corresponding derivative of lignoceric acid is given as 90°–91°C. Drake and Bronitsky reported the melting point of the *p*-phenylphenacyl ester of stearic acid to be 90°C.

From these results, the methyl esters of the saturated acids from the starch lipids appear to consist of 70.5% methyl palmitate, 25.9% methyl stearate, and 3.9% of an acid (or acids) of molecular weight higher than that of stearic acid and which appears to be arachidic acid, although the small sample obtained was not sufficient to allow for definite identification.

4. The unsaponifiable fraction.—The percentages of the free and combined sterols in the starch lipids were determined according to the method given by Abderhalden (1925), using digitonin as the precipitating agent. The factor 0.2563 was used to calculate sterol from digitonin steride. This is in accordance with the findings of Sandqvist and Bengtsson (1931), which were confirmed by Windaus, Werder, and Gschaider (1932).

Unsaponifiables amounted to 8.0% of the total lipids (methanol extract). Sterol determination on this unsaponifiable fraction showed it to be 75.8% sterols (6.07% of the total lipids). Free sterols, present in the unhydrolyzed lipids of the methanol extract of the starch, amounted to 1.78% of the total lipids or 29.3% of the total sterols present. The remainder of the sterols were present in combined form in the original lipids.

Summary

Corn starch contains approximately 0.65% of lipids. Starch separated from degerminated corn was found to have the same percentage of "fatty material" as commercially prepared starch; hence the lipids of starch are not present as a result of contact with lipid material during milling. Not a very high percentage of the lipids could be removed from starch by methanol extraction in a Soxhlet extractor (cold solvent) but by using the same solvent and an arrangement as described by Stokes (hot solvent) 93% to 95% of the lipids could be removed. This methanol extract is not made up entirely of fatty acids or lipids. Methanol removed the unsaturated acids more rapidly than the saturated. A portion of the phosphorus of corn starch is removed by methanol extraction. An extraction that removed 95% of the lipids lowered the phosphorus content of the starch from 0.016% to 0.010%. Only a small portion of this phosphorus can be attributed to phosphatides. Small amounts of protein are also removed from starch by methanol extraction.

Lipid samples for analysis were prepared as the ether-soluble material in the methanol extract of the starch, and as the ether-extractable material liberated from the starch by acid and by diastatic hydrolysis. For samples obtained from the methanol extract and the acid hydrolysis, the proportions of fatty acids were the same, while in the case of diastase hydrolysis, it was not possible to liberate all of the fatty acids from the starch. The lipids in the methanol extract are made up largely of free fatty acids. However, a positive test for glycerol was obtained on the lipids obtained by the methanol extraction.

The components of the lipid material (ether soluble) in the extract are: fatty acids, 92.0%, of which 88.0% are free in the starch; unsaponifiable, 8.0%, of which 75% is sterols (30% of total sterols is free); and phosphatides, 0.7%.

The fatty acids liberated from starch by acid hydrolysis were composed of palmitic 21.2%, stearic 7.8%, undetermined saturated acids 1.0%, oleic 37.7%, linolic 31.1%, and linolenic 1.2%. The unsaturated fatty acids were separated and partially identified by the bromination procedure. Oleic acid was also identified by purification through the lithium salt. Acids with unsaturation greater than three double bonds were not found.

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MICROSCOPIC EXAMINATION OF DEVELOPING CORN STARCH [1]

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(Received for publication December 13, 1940)

The microscopic examination of developing starch granules involves problems of a botanical nature, but is of interest to the chemist as it may aid him in his attempts to solve the mysteries of the chemical constitution and the physical and chemical behavior of starch. The present paper reports some observations made on the starch granules from corn kernels at different stages of maturity.

Starch for this study was obtained from a yellow field corn, 403 hybrid, developed by the Agronomy Division at the University of Minnesota, and grown on the University Farm. The shoots were marked with string when the visible silks were three or more in number and two centimeters or less in length. The first ears were picked 15 days after marking. Corn at seven different stages of maturity was harvested: 15, 22, 29, 36, 43, 50, and 57 days after silking.

The kernels were cut from the cob for the 15, 22, and 29-day samples, ground in a meat chopper, and screened through a 60-mesh screen. The material remaining on the screen was washed, the washings being added to the first material that passed through the screen. The combined suspensions were allowed to settle overnight at 4°C., the liquid was siphoned off, and the corn gluten was removed from above the starch cake. The starch was washed two times by resuspending in water and centrifuging in a cup centrifuge. The protein layer above the starch was cut away each time with a spatula. The cake was again suspended in water and then run through No. 17 bolting silk. The bolted suspension was centrifuged, the starch cake being suspended in water and centrifuged three times. The starch was then removed to pans, blotted as dry as possible, broken into small pieces, and air-dried in thin layers.

The 36, 43, 50, and 57-day samples of corn were firm enough to be shelled. It was necessary to soak the 43, 50, and 57-day samples in SO_2 water (0.25% SO_2) at 60°C. for 24 hours in order to be able to process the kernels. After this steeping, the steep water was poured off and the corn was run through a meat chopper, mixed with water and screened, and then carried through the same process as described for the earlier samples.

[1] Paper No. 1866, Scientific Journal Series, Minnesota Agricultural Experiment Station. From part of a thesis presented by James W. Evans to the faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June, 1940.

[2] Present address: Union Starch and Refining Co., Granite City, Illinois.

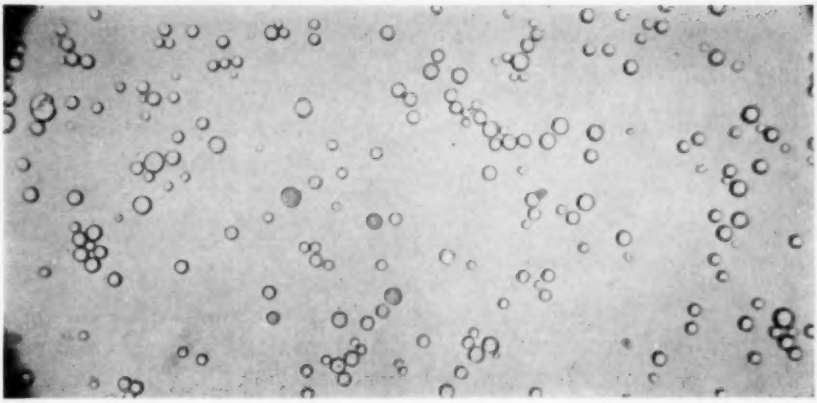

Fig. 1. Photomicrograph of corn starch 15 days after silking (270 ×).

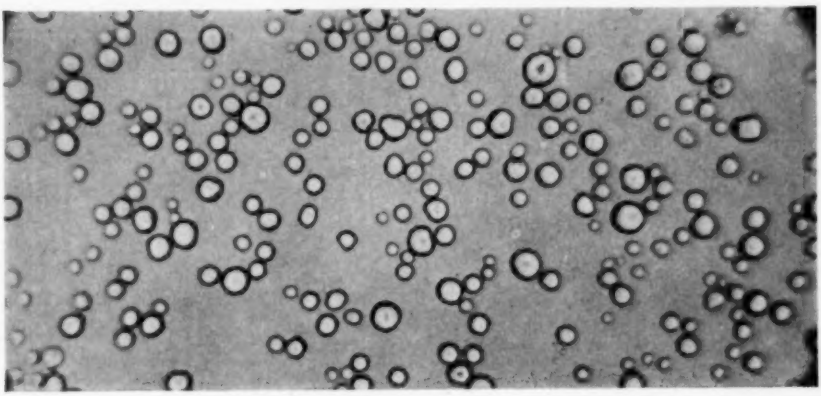

Fig. 2. Photomicrograph of corn starch 22 days after silking (270 ×).

The starches were suspended in water and photographed (magnification 410 ×). Photomicrographs of starch prepared from corn at four successive stages of maturity are reproduced in Figures 1, 2, 3 and 4.

The average size of mature corn starch granules is 10 to 25 microns, but deviations in both directions are common. The granules are usually polygonal but many round ones are found.

The photomicrographs of the starch from different aged kernels show a marked difference in size and shape of the starch granule. The outlines of the granules from the 15-day corn (Fig. 1) are round and the granules are much smaller than those from more mature kernels. Starch at this early stage cannot be recognized by microscopic examination as corn starch. At 22 days (Fig. 2) much larger granules

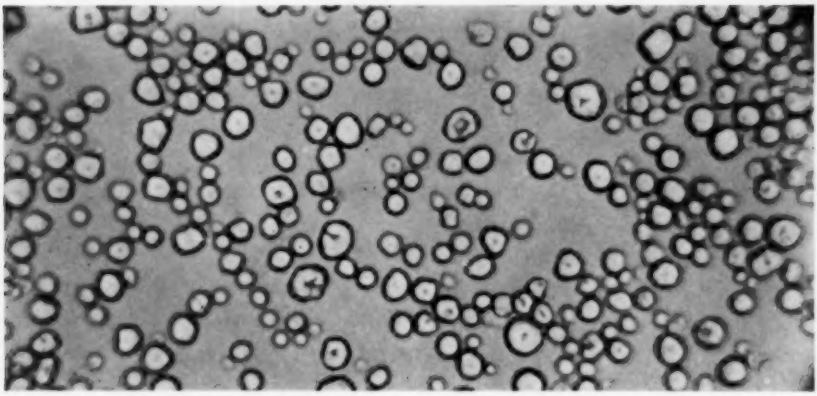

Fig. 3. Photomicrograph of corn starch 29 days after silking (270 ×).

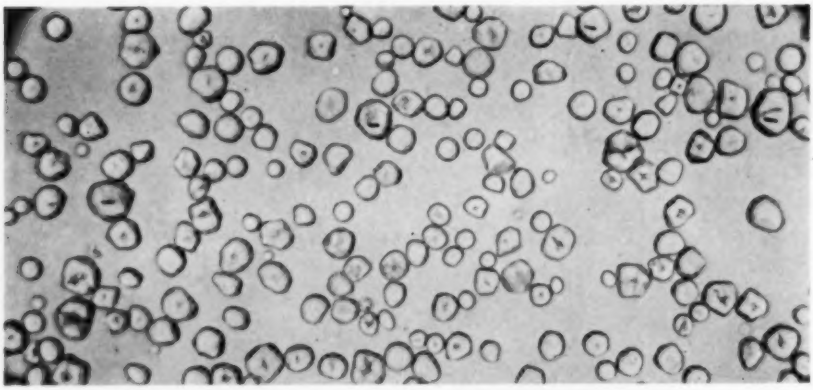

Fig. 4. Photomicrograph of corn starch 36 days after silking (270 ×).

are present and some of these have polygonal outlines. The starch from 29-day corn (Fig. 3) contains still larger granules, which have increased in relative number in the 36-day sample (Fig. 4). The influence of packing on the shape of the granules can be observed in the photomicrograph of the 36-day sample. The marked granules (-) in Figure 4 (36-day sample) show the indentations made on them by other granules that have been packed around them. There was very little difference between the 36- and 57-day samples.

The starches were observed between crossed nicols and all samples gave a well defined cross.

The photomicrographs presented in this paper show that the starch granules from corn increase in size as the kernel becomes more mature and that their outlines change from circular to polygonal. This change in shape is due to the packing of the granules as the kernel fills and becomes harder.

THE FATTY ACID COMPOSITION OF THE LIPIDS OF CORN STARCH AT VARIOUS STAGES DURING THE DEVELOPMENT OF THE CORN KERNEL[1]

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(Received for publication December 13, 1940)

Several studies have been made of the fatty acids associated with the starch from mature corn kernels (Taylor and Nelson, 1920; Taylor and Lehrman, 1926; Taylor and Werntz, 1927; Taylor and Sherman, 1933; Schoch, 1938). There are no reports in the literature of a study on the fatty acids and other lipids in corn starch prepared from corn kernels at different stages of development. Since information of this type may be of value in leading to an understanding of the formation of starch, the present paper reports the results of such a study.

The starch was separated from corn kernels of various degrees of maturity: namely, 15, 22, 29,36, 43, 50 and 57 days after silking. The procedure employed has been described in the preceding paper on "Microscopic Examination of Developing Corn Starch" (1941).

Data with regard to the starch yields obtained are given in Table I.

TABLE I

DATA ON STARCH YIELDS OBTAINED FROM CORN KERNELS HARVESTED AT VARIOUS STAGES OF DEVELOPMENT

Days after silking	No. of ears	Weight with husk	Weight without husk	Weight of cut or shelled corn	Starch
		lbs.	*lbs.*	*lbs.*	*lbs.*
15	842	732	234	180.5	3.0
22	227	225	167	80.3	7.1
29	122	113	78	54.0	7.4
36	100	99	80	49.3	10.6
43	69	—	45	36.0	12.0
50	86	—	58	47.5	16.2
57	132	—	88	72.1	26.1
Total	1578		750	519.7	82.4

Analysis of the starches for protein, ether extractable substances, lipids obtainable after acid hydrolysis, and phosphorus content are shown in Table II.

[1] Paper No. 1867, Scientific Journal Series, Minnesota Agricultural Experiment Station. Condensed from part of a thesis presented by James W. Evans to the faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June, 1940.

Eight hundred- to 1500-gram samples of starch, obtained from corn kernels at each of the various stages of maturity, were hydrolyzed by acid, according to the method of Taylor and Nelson (1920), and the lipids were extracted from the residue with ether. Precautions were taken to prevent oxidation of the lipids by carrying out the hydrolysis and extraction in an atmosphere of nitrogen and drying the extracted material in a vacuum in a stream of nitrogen.

TABLE II

ANALYSIS OF STARCH OBTAINED FROM CORN KERNELS HARVESTED AT VARIOUS STAGES OF DEVELOPMENT

(Values given as percentages of the starch on dry-weight basis)

	Days after silking						
	15	22	29	36	43	50	57
Protein (N×6.25)	0.338	0.175	0.263	0.200	0.206	0.200	0.194
Ether extract	0.06	0.05	0.06	0.06	0.05	0.05	0.06
Phosphorus	0.012	0.015	0.018	0.020	0.020	0.019	0.019
Fat "by hydrolysis"	0.49	0.23	0.31	0.49	0.53	0.54	0.54

The lipid material was separated into the saponifiable and unsaponifiable fractions. The fatty acids were liberated from the saponifiable fraction and separated into saturated and unsaturated portions (Twitchell method). The constituent unsaturated fatty acids were calculated by using the iodine (Wijs) and thiocyanogen values of the total fatty acids in Kaufmann's and Keller's equations.

Saturated fatty acids from the 57-day sample were separated by fractional distillation of their methyl esters and were identified by their neutralization equivalents and by their bromphenacyl and *p*-phenylphenacyl esters.

The methods of analysis were the same as those described in a preceding paper on the "Lipids of Corn Starch" (1941).

Results of the lipid analyses are given in Table III.

The starch from corn 15 days after silking contained 0.49% "fat by hydrolysis." By 22 days this had decreased to 0.23%, after which it again increased to 0.53% at 43 days, thereafter remaining essentially constant. The decrease in lipid content at 22 days cannot be entirely attributed to dilution due to the rapid increase of starch in the kernel at this period because the proportions of the constituent fatty acids also change. The saturated fatty acids increased from 13.9% of the total fatty acids at 15 days to 27.0% at 22 days, then to approximately 30% at 36 days. From this time to 57 days the relative amount

remained practically constant. A probable explanation for the increase in saturated fatty acids lies in the preferential adsorption of saturated fatty acids by starch.

TABLE III

ANALYSIS OF THE LIPIDS FROM STARCH OBTAINED FROM CORN KERNELS HARVESTED AT VARIOUS STAGES OF DEVELOPMENT

(All values are on dry-weight basis)

	Days after silking						
	15	22	29	36	43	50	57
Fat "by hydrolysis" (% *of starch*)	0.49	0.23	0.31	0.49	0.53	0.54	0.54
Acid value	190.7	177.3	186.1	195.5	196.0	195.4	196.1
Iodine value:							
Rosenmund-Kuhnhenn	117.8	87.8	86.4	83.0	83.3	82.7	83.0
Wijs	127.5	99.9	96.2	88.3	89.3	88.8	89.1
Thiocyanogen value	67.6	55.3	54.2	58.1	57.2	57.9	57.3
Unsaponifiable matter (% *fatty material "by hydrolysis"*)	5.0	13.2	8.0	5.2	5.1	4.7	5.3
Total fatty acids (% *fatty material*)	94.8	86.4	91.6	94.0	94.9	94.6	94.0
Acid value	201.1	204.2	202.0	203.0	203.1	202.7	202.5
Mean molecular weight	279.0	278.1	277.8	276.2	276.2	276.7	277.0
Iodine value:							
Rosenmund-Kuhnhenn	104.0	89.1	88.0	87.1	86.6	86.0	86.9
Wijs	114.4	97.1	96.2	94.6	93.8	93.6	93.9
Thiocyanogen value	78.1	67.1	65.6	64.2	63.7	63.1	63.6
Saturated fatty acids:							
(Twitchell) (% *total f.a.*)	13.9	27.0	28.4	30.1	30.8	31.2	30.8
Iodine value	0.8	1.0	1.1	0.7	0.9	1.0	0.8
Mean molecular weight	268.3	268.8	267.8	269.2	269.0	268.2	268.8
Palmitic (% *total f.a.*)	—	—	—	—	—	—	22.0
Stearic (% *total f.a.*)	—	—	—	—	—	—	7.8
Undetermined (% *total f.a.*)	—	—	—	—	—	—	1.0
Unsaturated fatty acids (by diff.) (% *total f.a.*)	86.1	73.0	71.6	69.9	69.2	68.8	69.2
Mean molecular weight	280.7	281.0	279.9	280.0	280.3	279.2	280.5
Iodine value:							
Rosenmund-Kuhnhenn	123.0	122.4	122.8	123.9	124.4	123.6	123.9
Wijs	132.1	131.8	132.4	133.5	134.1	133.6	134.1
Oleic acid (% *total f.a.*)	46.2	40.1	38.0	36.5	36.2	35.3	36.0
Linolic acid (% *total f.a.*)	39.4	31.5	32.5	32.1	31.6	32.3	31.9
Linolenic acid (% *total f.a.*)	0.5	1.4	1.1	1.3	1.4	1.2	1.3
Unsaponifiable matter (% *of fatty material from methanol extract*)	—	—	—	—	—	—	7.5
Sterols (% *unsaponifiable matter*)	—	—	—	—	—	—	75.7
Total sterols (% *fatty material*)	—	—	—	—	—	—	5.68
Free sterols (% *fatty material*)	—	—	—	—	—	—	1.84
Combined sterols (% *fatty material*)	—	—	—	—	—	—	3.84

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CHANGES IN THE BIOCHEMICAL COMPOSITION OF THE CORN KERNEL DURING DEVELOPMENT [1]

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(Received for publication January 31, 1941)

A survey of the literature indicates that relatively few studies have been made concerning the variations in many of the biochemical constituents of the corn kernel, during the developmental stages. Lampe and Meyers (1925) made a study of the carbohydrate storage in the endosperm of sweet corn. Lampe (1931) reported the results of a microchemical and morphological study of the developing endosperm of maize. Zeleny (1935) traced the distribution of nitrogen in the corn kernel at different stages of maturity. The present paper reports the results of analyses of corn kernels at various stages of maturity in which moisture, crude protein, crude fiber, starch, sugars, ash, and ether extract were determined on the samples. Iodine values and free fatty acids were determined on the ether extract.

Preparation of Samples

Samples of a yellow field corn, 403 hybrid, were collected from the fields of the University Farm at St. Paul, Minnesota, at 15, 22, 29, 36, 43, 50, and 57 days after silking. This was accomplished by marking the shoots with string when the silks were 2 cm. or less in length. The lower limit was taken when 3 silks were visible. After the elapse of the desired time, the ears were picked and the kernels were removed from

[1] Paper No. 1868, Scientific Journal Series, Minnesota Agricultural Experiment Station. Condensed from part of a thesis presented by James W. Evans to the faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June, 1940.

[2] Present address: Union Starch and Refining Co., Granite City, Illinois.

the cob immediately. A small sample was taken for moisture determinations, and the remainder dried in a vacuum oven at 70°C. for 10 hours and stored in the refrigerator at 5°C. Whole kernels were selected and ground in a mortar to obtain material for the above-mentioned determinations.

Fifteen days after silking, the corn was very green, the kernels were about one-fifth mature size, and the silks were still quite green. In the 22-day sample the kernels were nearly full grown, still very "milky," and the silks were brown. The kernels were starting to dent in the 29-day sample and were only slightly "milky." The husks were just start-

TABLE I

COMPOSITION OF FRESHLY HARVESTED KERNELS

	Days after silking						
	15	22	29	36	43	50	57
Moisture, %	85.4	72.6	60.2	47.6	42.1	40.6	40.0
Dry substance, %	14.6	27.4	39.8	52.4	57.9	59.4	60.0
Crude protein ($N \times 6.25$), %	2.82	3.83	4.76	6.19	6.70	6.90	6.95
Starch, %	3.3	16.0	28.3	37.6	41.4	42.7	43.0
Sugars (reducing) as:							
Dextrose, %	0.77	0.96	0.79	0.42	0.43	0.48	0.44
Sucrose, %	0.56	0.77	0.93	1.26	1.36	1.43	1.40
Crude fiber, %	1.1	1.0	0.9	0.9	1.0	1.0	1.0
Ether extract, %	0.55	1.17	2.03	2.72	3.02	3.08	3.10
Ash, %	0.49	0.74	0.79	1.01	1.05	1.06	1.07

ing to turn brown. By 36 days the kernels were all dented and the husks were browner, and in 50 days the husks were dry and were bursting open.

Methods

Moisture content was determined at the time of harvesting by first drying the samples in a vacuum oven at 70°C. for 4 hours, then grinding the samples, and drying again in a vacuum oven at 100°–105°C. to constant weight. Protein (Kjeldahl-Gunning-Arnold method, using copper sulfate as a catalyst), ash, crude fiber, starch (diastase method with subsequent acid hydrolysis), reducing sugars, and sucrose (acid hydrolysis at room temperature), were all determined by official A.O.A.C. methods (1935).

In order to obtain the ether extract for iodine value and free fatty acid determinations, dried corn was extracted with anhydrous ether in a Soxhlet extractor. The ethereal solution was dried over anhydrous sodium sulfate, filtered, and made up to a definite volume with anhydrous ether. The amount of extracted material was determined by weighing

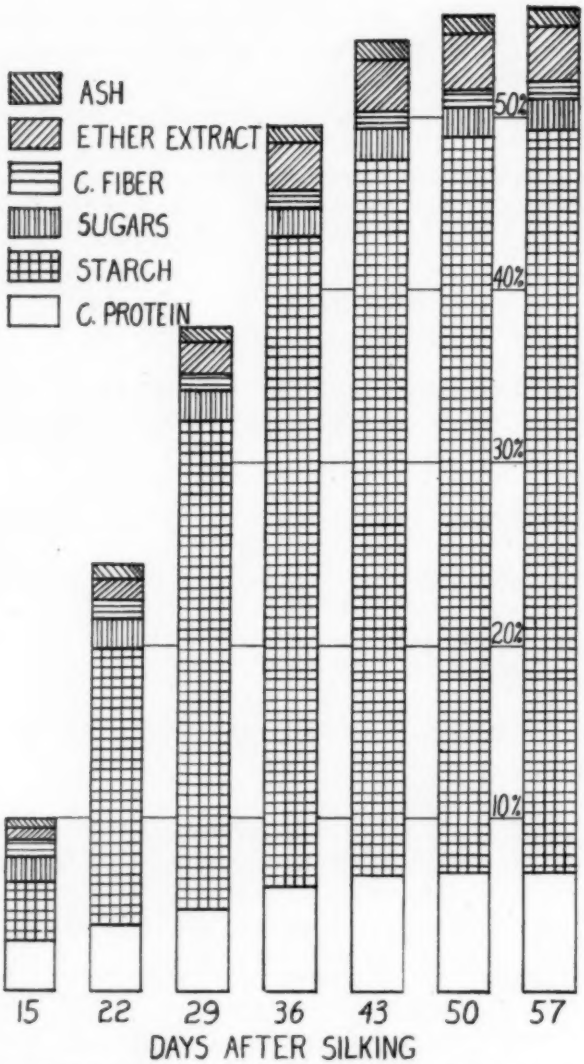

Fig. 1. Composition of corn kernels at various stages of growth, based on corn as harvested.

the residue after evaporating the ether from an aliquot. Iodine values (Wijs) and acid numbers (by A.O.A.C. methods) were obtained on aliquots from which the ether had been evaporated under diminished pressure in a stream of nitrogen, the temperature never exceeding 40°C.

Results and Discussion

The results of the analyses, reported on the basis of the corn kernels as harvested, are shown in Table I. These results are shown graphically

in Figure 1. These data show that there was a steady increase in the amounts of crude protein, starch, sucrose, ether extract, and ash in the corn kernels during their development. There was an increase in the percentage of reducing sugars from 15 to 22 days after silking, then a decrease to a more or less constant value after 36 days. There was little change in the percentage of crude fiber in the kernels.

TABLE II

COMPOSITION OF KERNELS ON DRY MATTER BASIS

	Days after silking						
	15	22	29	36	43	50	57
Crude protein ($N \times 6.25$), %	19.28	13.99	11.96	11.82	11.57	11.61	11.59
Starch, %	22.8	58.3	71.0	71.8	71.5	71.8	71.6
Sugars (reducing) as:							
Dextrose, %	5.24	3.49	1.99	0.80	0.75	0.81	0.74
Sucrose, %	3.80	2.82	2.34	2.40	2.35	2.41	2.33
Crude fiber, %	7.2	3.6	2.2	1.7	1.7	1.7	1.7
Ether extract, %	3.78	4.26	5.11	5.19	5.22	5.18	5.17
Ash, %	3.33	2.71	1.99	1.92	1.82	1.78	1.78
Undetermined, %	34.6	10.8	3.4	4.3	5.1	4.7	5.1

The above results of the analysis of the biochemical composition of corn kernels at various stages of development, calculated to the dry-substance basis, are given in Table II. These results are shown graphically in Figure 2. The percentages of starch and ether extract increased steadily to 36 days, at which time they reached a constant value. Crude protein, crude fiber, sugars, and ash decreased as the seed matured. These reached a constant value in about 43 days.

TABLE III

CHARACTERISTICS OF FAT FROM CORN KERNELS AT DIFFERENT STAGES OF MATURITY

	Days after silking						
	15	22	29	36	43	50	57
Ether extract (dry-substance basis), %	3.78	4.26	5.11	5.19	5.22	5.18	5.17
Iodine value (Wijs)	100.8	112.2	123.0	127.7	127.3	126.9	127.1
Acid value	67.2	58.8	54.5	23.5	13.2	13.5	13.4
Free fatty acids as oleic (% ether extract)	33.7	29.6	27.4	11.8	6.7	6.8	6.8

Iodine values (Wijs), acid values, and free fatty acids were determined on the ether extracts from the corn samples. The results are shown in Table III. The iodine values (Wijs) of the ether extracts of

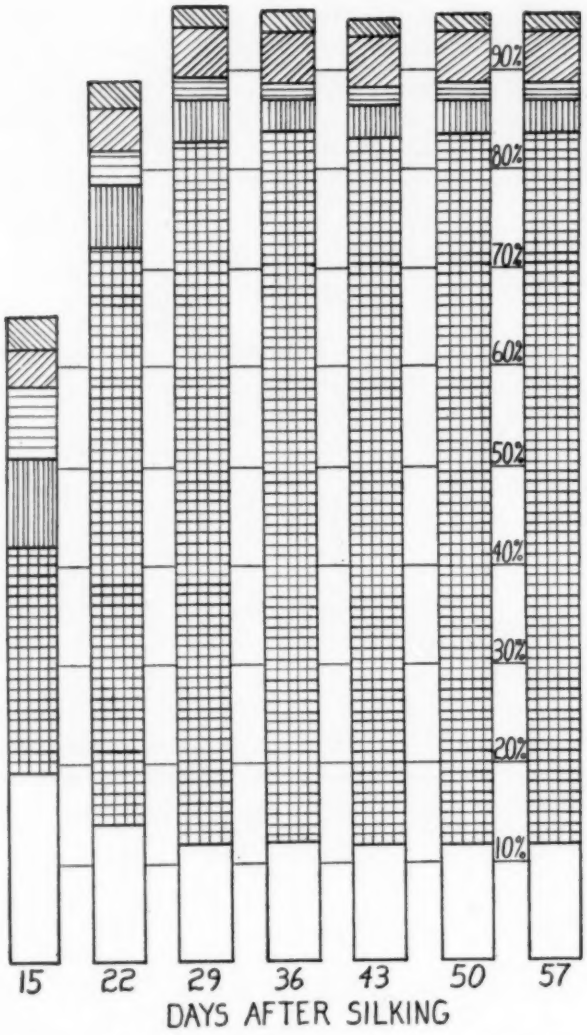

Fig. 2. Composition of corn kernels at various stages of growth, dry-substance basis. (See Fig. 1 for interpretation.)

corn kernels at successive stages of growth increased from 100.8 at 15 days after silking to 127.7 at 36 days. It appears that oil formation sets in at an early stage and that the nature of the oil progressively changes, becoming more and more unsaturated in character. The percentages of free fatty acids in the ether extracts, calculated as oleic acid, decreased from 33.7% at 15 days to 6.7% at 43 days and remained constant thereafter.

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A MODIFIED THIOCHROME METHOD FOR THE ESTIMATION OF VITAMIN B_1 IN WHEAT AND ITS PRODUCTS

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(Received for publication February 21, 1941)

In the past few months a great deal of attention has been centered on vitamin B_1 in flour and bread. Because of this interest it has been necessary for flour milling laboratories to consider methods for its estimation. Methods of choice should be adaptable for routine analysis and preferably not require unduly expensive equipment. In the literature a number of methods are described, but relatively few satisfy these particular requirements.

There are several biological tests. The rat-growth method is, at present, accepted as standard, but few laboratories have facilities for this type of work. Schopfer's method (1935) is based on the growth of a fungus in the presence of vitamin B_1, but Sinclair (1938) showed it to be not entirely specific. The rate of alcoholic fermentation is powerfully stimulated by vitamin B_1, and Schultz, Atkin, and Frey (1937) used this as a basis for a vitamin assay. They developed a fermentation test and later extended it to a microdetermination (1939). However, it appeared to the present authors that with this method there are many other materials present besides the vitamin, which could conceivably have some action on the fermentation rate. For that reason they have preferred, if possible, to use a method based on some quantitative chemical reaction of the vitamin.

Prebluda and McCollum (1939) first proposed the use of diazotized *p*-aminoacetophenone which forms a purple-red compound with vitamin B_1, the intensity of the color being a measure of the vitamin concentration. Melnick and Field (1939a, b, c) studied this method,

and extended it to the estimation of phosphorylated thiamin by incubation with yeast phosphatase. To remove materials that might interfere with the reaction, the vitamin is adsorbed on zeolite.

Jansen (1936) pointed out that vitamin B_1 could be measured quantitatively by conversion to thiochrome, a strongly fluorescent material, by oxidation with alkaline ferricyanide. A number of modifications of this test have been devised. Hennessy and Cerecedo (1939) used an enzyme prepared from defatted beef kidney in acid solution for extraction, and subsequent adsorption on Decalso. They measured the fluorescence photoelectrically. Wang and Harris (1939) concluded that the intensity of fluorescence could be estimated equally well by titrating a standard thiochrome solution into a blank and comparing visually. These workers removed pigments and other interfering materials from urine by washing the extracts prior to oxidation with isobutyl alcohol. Pyke (1938) stated that vitamin B_1 was associated in some materials with protein, and for that reason (1939) employed pepsin digestion followed by taka-diastase to liberate any vitamin that might be present in the phosphorylated form. Oxidation was carried out on the unpurified extract and the fluorescence estimated visually.

When considering chemical methods for vitamin B_1 determinations, it becomes apparent that two difficulties are inherent in all of them. Extraction presents a problem due to the fact that the vitamin can exist free and as phosphoric acid esters, and methods must take this into account in order to give a true measure of vitamin potency. Secondly, the listed reactions of the vitamin are influenced by the presence of certain interfering materials. At present little information is available regarding the nature of these interfering compounds, and it is probable that extracts of some materials will contain them while others do not. Hence, the selection of a method should be based on the ease and efficiency with which these problems can be overcome.

Pyke's method appeared the most promising, for the double enzyme treatment seemed to be a logical way to liberate the vitamin. He reported results in good agreement with those of other investigators without resorting to adsorption for freeing the vitamin of interfering materials. The present authors wished to avoid methods employing adsorption because previous experience has shown them to be highly sensitive to manipulation. Furthermore Wang and Harris (1939) and Pyke (1939) reported visual matching of fluorescence with an accuracy comparing favorably with methods involving photoelectric instruments. This is an important item since the latter type of instrument is generally very costly. Thus it was decided to use this procedure for wheat and its products.

Pyke's Thiochrome Method

The thiochrome method of Pyke (1938) is as follows: "20 g. of foodstuff is finely minced or powdered. A solution of 0.1% pepsin in 0.33% hydrochloric acid is added and the volume made up to 97.4 cc. The mixture is incubated overnight at 37°. 2.6 cc. of normal sodium hydroxide and 100 mg. of taka-diastase are added and the incubation continued for another 5 hours. The extracts are then centrifuged. Two 3-cc. aliquots are pipetted into graduated cylinders in which the following reagents are kept stirred by means of a stream of nitrogen. The first cylinder contains 2 cc. of methyl alcohol, 1 cc. of 30% sodium hydroxide, and 1 cc. of 1% potassium ferricyanide. The second cylinder contains 2 cc. of methyl alcohol and 1 cc. of 30% sodium hydroxide only. The nitrogen is kept bubbling for one minute. The solutions are then made up to 10 cc. with water, 13 cc. of isobutyl alcohol is added to each, and they are well mixed. Shaking should not be too vigorous, or troublesome emulsions may be formed. When the supernatant layers become clear, 10-cc. aliquots are pipetted into uniform test tubes, and 1 cc. of methyl alcohol is added to each of them. The tubes are matched by holding them side by side at an angle of 45° against a nickel oxide filter arranged in a vertical position to transmit the ultraviolet light of a mercury vapor lamp. A standard solution of thiochrome is added, 0.1 cc. at a time, to the aliquot prepared without the use of ferricyanide, until the color and intensity of the fluorescence of the two tubes has reached the best possible match."

Preliminary experiments on wheat products proved the following in regard to this method:

1. Extraction.—Fineness of grinding is important. The material should be ground as fine as the available equipment permits. In preparing samples for analysis in this laboratory all were ground to pass at least a 50-mesh sieve.

Making up to a total volume of 97.4 cc. presupposes that all materials occupy the same volume. Bran, for example, occupies a considerably larger volume than the same weight of ground wheat, and hence would require less acid-pepsin solution. A definite volume of acid solution should therefore be used.

The greatest activity of pepsin is at pH 2.0 to 2.5; therefore, instead of adding a definite strength of acid, the acidity should be so adjusted that pH of the suspension is in the correct range. This will allow for any differences in buffer value of the material being analyzed.

2. Presence of interfering materials.—It was found that the extracts from some materials may be oxidized directly after extraction, but on the whole, interfering compounds must be removed. Particularly is this important with wheat germ; otherwise only 50% to 60% of the

true figure is realized. According to McFarlane and Chapman (1941) this can be conveniently accomplished by making the solution up to 50% with alcohol and heating to boiling. This procedure clarifies the extracts and reduces their organic matter content.

However, the use of an alcoholic extract resulted in even greater color in the blank used for the thiochrome titration, and made matching of the two tubes more difficult. McFarlane and Chapman (1941) found that this could be remedied by the addition of hydrogen peroxide, which largely destroyed the foreign color yet affected no oxidation of the vitamin. This confirmed the work of Kinnersley and Peters (1928), who failed to oxidize any thiamin to thiochrome with hydrogen peroxide. The interfering color here mentioned is probably due to the presence of flavone pigments, which are intensely yellow in alkaline solution.

3. Oxidation to thiochrome.—Pyke's method calls for a 3-cc. aliquot of extract to be used for oxidation, but this is not always suitable. When analyzing bread that is relatively low in vitamin compared with other cereal products, the final extract is only feebly fluorescent, whereas a 5-cc. aliquot results in a more easily matched extract. Wheat germ, on the other hand, may contain 1,000 I.U. or more per 100 g., and in that case a 3-cc. aliquot yields an extract that is too bright for convenient matching. Hills (1939) proved that the relation of concentration to fluorescence is not linear at high concentrations. Therefore, the size of aliquot should be adjusted so that the concentration in the titrated extract is between 0.4 and 3.0 micrograms of vitamin B_1.

It was found that insufficient ferricyanide results in only partial oxidation, whereas an excess destroys thiochrome. To a great extent, the amount added is dependent on the amount of organic material in solution, for a large part of the ferricyanide is used in oxidation of this material. Thus the quantity of ferricyanide may not be the same for all classes of materials and should be carefully checked.

It was found also that only partial oxidation may occur if the sodium hydroxide and the potassium ferricyanide are mixed prior to the addition of the extract aliquot. This was first noticed by a fading of the green color on standing, due no doubt to the reduction of the ferric to the ferrous salt in the presence of strong alkali. This was overcome by always adding the 30% sodium hydroxide last.

Since this work was completed Booth (1940) has published a paper in which he points out this criticism of Pyke's procedure regarding the order for the addition of the reagents.

The Modified Thiochrome Method [1]

On the basis of findings discussed above, a modified method has been developed, the details of which are presented as follows:

Five grams of finely ground material (through 50-mesh or finer) are weighed into a 125-cc. Erlenmeyer flask, 25 cc. of hydrochloric acid solution containing 25 mg. pepsin is added, and the mixture vigorously shaken. The acid should be of such a strength that the pH of the suspension is approximately 2.0. The sample is incubated overnight at 37°C. The pH of the mixture is then adjusted to 4.7 with approximately normal sodium hydroxide, 25 mg. of taka-diastase is added, and the incubation continued for a further four to five hours. Twenty-five cc. of c.p. methyl alcohol is added and the mixture is heated to boiling under a reflux condenser, cooled, and centrifuged at approximately 2,000 rpm. for 10 minutes. Two aliquots (1 to 5 cc.) are pipetted into 25-cc. glass-stoppered graduated cylinders, containing 2 cc. of methyl alcohol (one for oxidation and one for blank). Three-tenths to 1.0 cc. of fresh 1% potassium ferricyanide is added to the "oxidation" cylinder only, and followed by 1 cc. of 30% sodium hydroxide to both. The cylinders are shaken for approximately one minute, then 1 cc. of 30% hydrogen peroxide is added, they are shaken again, and are then allowed to stand for two to three minutes. The solutions are made up to 10 cc. with water, and 13 cc. of isobutyl alcohol is added and well mixed. The separation of the two layers is accomplished by standing or by centrifuging at medium speed for one minute. Ten cc. of the clear supernatant liquid is pipetted into uniform test tubes containing 1 cc. of methyl alcohol, and these are used for matching.

The pH is best checked after two hours' standing, as the pH of some wheat products will shift appreciably during that time.

The volume of added alkali should be recorded, as the total volume of liquid enters into the final calculation.

No particular advantage is gained by substituting ethyl alcohol for methyl alcohol. However, if it is desired to use denatured alcohol, the result should be checked by means of a sample of known vitamin content.

According to Hills (1939), filtering may be substituted for centrifuging, provided the filter papers have first been subjected to continuous hot extractions with isobutyl alcohol for 24 hours; otherwise some adsorption of vitamin may result.

Glass-stoppered apparatus is preferred throughout, but if corks are used they should be covered with tinfoil to prevent extraction of fluorescent material from them.

[1] Since this manuscript was prepared, it has been found that the dilution of the sample aliquot with 2 cc. of methyl alcohol before the oxidation should be omitted, because this step introduces a serious error with flours of low B_1 content.

It is important that the potassium ferricyanide should be of c.p. quality.

Care should be exercised in the selection of the test tubes; otherwise very serious errors may be introduced. Those used during this study were supplied by the Rubicon Co., for use with the Evelyn Photoelectric Colorimeter. They have an inside diameter of 20 mm. and overall length of 175 mm.

Preparation of Standard Thiochrome

This solution should be prepared fresh daily as it is a relatively unstable compound. The solutions of the vitamin used in the preparation of the standard thiochrome, however, have been found to be stable over a period of months provided they are kept at a pH not higher than 5.0.

It has been found convenient to have two standard thiochrome solutions, the weaker for materials like bread and flour, and the stronger for bran and germ. One-cc. volumes of solutions containing 3.0 or 30.0 μ g. of vitamin B_1 are added to 2 cc. of methyl alcohol in a small separatory funnel. Two drops of 1% potassium ferricyanide are added, followed by 1 cc. of 30% sodium hydroxide, and thorough mixing. Ten cc. of isobutyl alcohol is added, and the mixture vigorously shaken. The aqueous layer is allowed to settle, drawn off, and discarded. One cc. of methyl alcohol is added and the volume made up to 15 cc. with isobutyl alcohol. One cc. of the weaker standard is equal to 0.2 μ g., and the stronger is equal to 2.0 μ g. of vitamin B_1.

The Fluorimeter

A schematic drawing of the apparatus is presented in Figure 1. An 85-watt mercury vapor lamp, with a polished reflector, is mounted approximately 15 cm. behind a nickel oxide filter in a ventilated box. The lamp is set horizontally and parallel to the filter in order to give even illumination for the test tubes directly in front. Many completely assembled units have the lamps at right angles to the filter, but with this arrangement the position of the test tubes is too critical. The test tubes are held side by side in sleeve holders that extend from the top of the tube to within 4 cm. of the bottom and hold them at an angle of 45° to the filter.

Matching is best accomplished by looking down, but at a slight angle so as to compare the fluorescence in the section of the tube from the bottom of the sleeve holders to within 1 cm. of the bottom of the test tubes. This eliminates error due to the variable thickness of the tube bottoms.

The titration of the standard thiochrome from a 10-cc. microburette should be made dropwise with shaking; otherwise errors can be introduced. If this operation is done too quickly, improper mixing results. Under these conditions the blank appears brighter than when

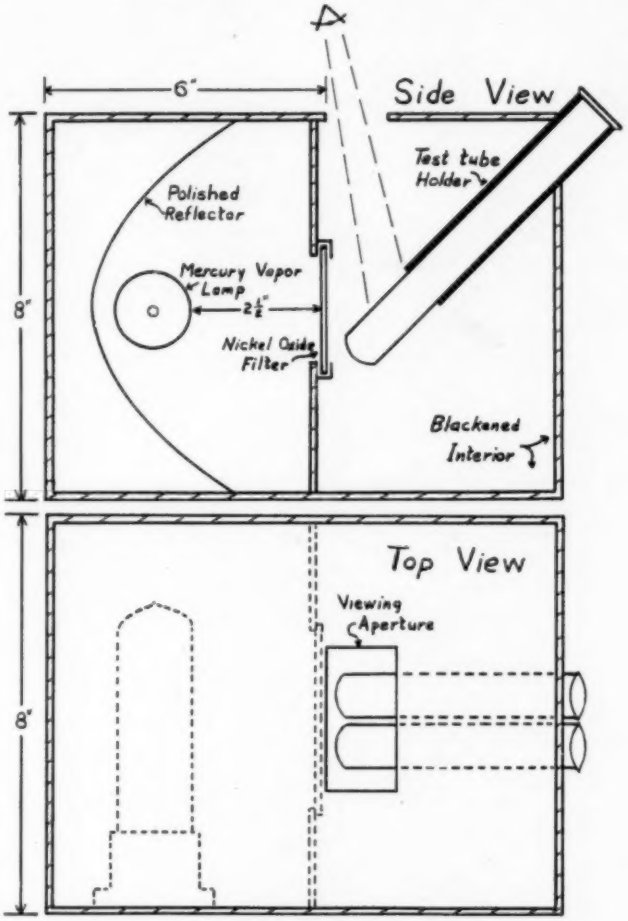

Fig. 1. Visual fluorimeter.

the thiochrome is evenly distributed in the mass of the liquid. The whole operation of matching should be conducted in a darkened room.

In this laboratory it has been the practice to begin an analysis in the late afternoon with the pepsin incubation. The following morning, the pH is readjusted, the taka-diastase is added, and the samples incubated till the early afternoon. Then the oxidations are made and the fluorescence judged. In this way a single operator may conveniently make eight determinations per day.

The following are typical calculations:

Aliquot = 3 cc.
Total vol. liquid = 55 cc.
Titration = 1.25 cc. (weaker standard)

The 3-cc. aliquot represents $3/55 \times 5 = 0.273$ g., original material, but 10 cc. of a total of 17 cc. was used for matching; therefore the 3-cc. aliquot actually represents $10/17 \times 0.273 = 0.161$ g. original material. Vitamin B_1 of sample $= 1.25 \times 0.2/0.161 = 1.56$ μ g. per g. or 52 I.U. per 100 g.

Replicability

As a check on the ability of a person to estimate fluorescence visually, varying amounts of the thiochrome standard were measured into test tubes and made up to 10 cc. with isobutyl alcohol. These were titrated in the usual way. The results in terms of vitamin B_1 are recorded in Table I.

TABLE I

DATA FROM TITRATION OF KNOWN AMOUNTS OF THIOCHROME
(Expressed as micrograms of vitamin B_1)

Actual concentration	Found	Difference
0.20	0.21	0.01
0.40	0.40	0.00
0.80	0.78	−0.02
1.60	1.66	0.06
3.00	3.02	0.02

These data confirm the statement of Wang and Harris (1939) that fluorescence can be measured accurately by visual means. However, it should be pointed out that these data do not represent the accuracy attainable with actual cereal extracts because of a faint color in the blank. The hydrogen peroxide treatment does destroy the bulk of the foreign color, but still a trace remains.

Two samples, a flour and a wheat, were analyzed in quadruplicate on successive days. Each extract was also treated in duplicate to indicate the variability within a single determination. These data are presented in Table II.

It appears from Table II that there is as great a variability between aliquots of the same extract as between different extracts. This would indicate that the oxidation process is one of the sources of error. Day-to-day variation also appears to exist, and this is probably due to preparation of the thiochrome standard used for the titration. Therefore particular attention should be paid to these operations. However, it can be concluded that the replicability compares very favorably with vitamin determinations in general.

TABLE II

REPLICATE ANALYSES OF FLOUR AND WHEAT ON SUCCESSIVE DAYS
(Expressed as International Units of vitamin B_1 per 100 g.)

Extract No.	1st day		2nd day	
	FLOUR			
1	71	73	73	75
2	73	73	77	75
3	75	75	78	78
4	75	75	77	75
Daily mean	74.4		76.0	
	WHEAT			
1	136	133	136	140
2	140	136	140	140
3	140	133	140	136
4	140	144	144	136
Daily mean	137		139	

Recovery Tests

As a further check on the method, known amounts of thiamin were added to a ground wheat and a flour and the whole procedure followed through in the regular way. The recoveries on the two samples were 97% and 97.5% respectively.

Comparison of Visual and Photoelectric Methods

The Department of Chemistry, Macdonald College, Quebec, kindly consented to collaborate with the authors to determine the variability between two investigators using visual and photoelectric fluorimeters Their instrument has been described by Froman and McFarlane (1940). These data are given in Table III.

TABLE III

ANALYSES BY TWO INVESTIGATORS USING VISUAL AND PHOTOELECTRIC FLUORIMETERS
(Results expressed as International Units of vitamin B_1 per 100 g. material)

	Visual—the authors	Photoelectric—McFarlane and Chapman
Bread	83.5	86
Second clear flour	200	202
Bran	225	215
Wheat germ	925	950

These data indicate good agreement between the two types of measurement, provided the visual matching has been made by an experienced operator. However, it should be pointed out that a certain amount of practice is necessary in order to obtain concordant results, but that is a prerequisite for any type of visual color matching.

Summary

With Pyke's thiochrome method as a basis, a new modified procedure for vitamin B_1 in cereal products has been described and precautions discussed. The method is satisfactory for wheat and its products, and is relatively rapid and easily adaptable for routine work. Because it utilizes a visual method for measuring the intensity of fluorescence, the apparatus required is inexpensive; however, the results compare well with those obtained with the more costly photoelectric instrument. Data on the replicability of the method and recovery of added thiamin have been presented.

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SODA REQUIREMENTS OF CRACKER FLOURS

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(Read at the Annual Meeting, May 1940)

In the cracker and biscuit industry the term "soda" is applied to sodium acid carbonate ($NaHCO_3$), and in this work whenever this term is used it also refers to sodium acid carbonate. The various commercial brands on the market are almost chemically pure (99.6% to 99.9% purity). To cracker bakers soda is known as a "chemical," but in the legend on the package soda is usually classified as a "leavener."

The Function of Soda in Fermentation and Baking

The terms "crackers," "soda crackers," and "oyster crackers" (also known as "sponge goods") include the products of various shapes and sizes such as: square (2-inch standard size and 2½-inch larger size), oblong (flake), round (wafer), and small round (oyster).

Crackers are made from fermented doughs of 18 to 23 hours' sponge and 3 to 5 hours' dough fermentation time with 0.2% to 0.5% yeast, 1.0% to 1.25% salt (usually 1.0%), 0.5% to 0.7% soda, 5.0% to 15.0% shortening, and 27% to 35% water (based on the flour as 100). In cookie and biscuit doughs soda combined with an acid base is chiefly used as a leavener, but this is not the case in crackers where soda is used alone with no acid base added. The primary function of soda in crackers is chemical and physico-chemical, rather than to provide a leavening action. As a result of the neutralization of the natural acidity present in the flour as well as the acidity created in the fermentation (in both sponge and dough), the desirable qualities of spring, tenderness, texture, grain, color, and bloom, are secured in the finished product.

The leavening action in cracker fermentation is largely due to the yeast, and on the other hand soda added after 18 to 23 hours of sponge fermentation has a great deal of retarding effect on fermentation and gas production in the dough stage. However, this retarding effect is due to the "free" soda which was not neutralized by the acidity created in fermentation and the natural acidity present in the flour; consequently the larger the amount of the "free" soda the greater is the retarding effect.

The retarding effect of soda on fermentation can be best seen if soda is omitted, when fermentation proceeds at a much faster rate than under the normal conditions, while if the normal amount of

soda is doubled, the fermentation is almost stopped with practically no gas production. Both these extreme cases may occur accidentally in commercial practice. The gas production due to the direct reaction of soda with the acids created in fermentation appears to be negligible as to its contribution to leavening of the finished cracker and is not considered at all in the commercial practice.

There is a certain school of thought who maintain that the main value of soda in baking is due to the leavening action according to the reaction whereby 2 $NaHCO_3$ is decomposed into Na_2CO_3, CO_2, and H_2O. However, this reaction does not occur because there is, and usually there should be, very little or none of the "free" soda (not neutralized) either in the well fermented doughs or in the finished product, if the pH is close to the neutral point or slightly on the alkaline side. $NaHCO_3$ decomposes at 270°C. (518°F.) (*Handbook of Chemistry and Physics*, 1937–38), whereas the baking temperatures of crackers are 232° to 288°C. (450° to 550°F.) with a very short baking time of three to four minutes. Furthermore, the inside temperature of the cracker in the oven during baking is slightly above the boiling point of water 104°C. (219°F.). Finally, the analysis of the baked cracker even with the pH above 8.1 did not show the presence of CO_3'' (Na_2CO_3).[1]

There is a rise in pH during baking, which Johnson and Bailey (1924) attributed to the conversion of the acid carbonate into the normal carbonate "and the loss of the acid reacting carbon dioxide from the dough during the baking process." The same authors also state that "These observations suggested that some reaction other than the bicarbonate to carbonate conversion was involved in effecting changes in hydrogen ion concentration during the baking process." Our tests have shown that there is a similar rise in pH in the acid cracker (for example pH 5.8 of the unbaked cracker was changed to 6.6 after baking), which hardly can be attributed to the conversion. On the other hand we agree on the points that "the loss of the acid-reacting carbon dioxide from the dough during the baking process" is one of the factors responsible for the increase in pH during baking and also that there are still other changes, probably very complex, which will require further study.

Selection and Blending of Cracker Flours

We have for many years made comparisons for quality of crackers in the different plants operated in various sections of the United States. The crackers compared were of good grade (standard, the

[1] The test for CO_3'' with phenolphthalein was negative, while methyl orange showed the presence of HCO_3' (*Technical Methods of Analysis*, by R. C. Griffin, 1927, pp. 30–31).

TABLE I

ANALYTICAL AND PRODUCTION DATA ON FLOURS USED IN VARIOUS PLANTS

Plant	Dough	Flour brand[1]	Ash[2]	Protein[2]	Viscosity[2,3]	Relative percentage of flours	Relative quantity of protein	Ratio of sponge to dough flours	Relative quantity of protein in sponge, dough, and total flours	Soda per one-barrel batch	Percentage of soda based on flour	pH of the cracker
			%	%		%	%	%	%	*lbs.*	%	
A	Sponge	1	0.41	9.50	76	20.00	1.90					
		2	0.42	9.10	60	40.00	3.64	60.00	5.54			
	Dough	2	0.42	9.10	60	40.00	3.64	40.00	3.64			
									9.18	1.17	0.60	7.5
B	Sponge	3	0.40	9.50	74	30.00	2.85					
		4	0.41	9.70	80	30.00	2.91	60.00	5.76			
	Dough	3	0.40	9.50	74	20.00	1.90					
		5	0.38	7.70	24	20.00	1.54	40.00	3.44			
									9.20	1.22	0.62	7.4
C	Sponge	6	0.41	9.50	68	14.29	1.36					
		7	0.41	9.80	86	42.85	4.20	57.14	5.56			
	Dough	8	0.40	8.10	33	14.29	1.16					
		9	0.43	9.00	58	28.57	2.57	42.86	3.73			
									9.29	1.05	0.54	7.8
D	Sponge	4	0.41	9.70	80	32.00	2.75					
		10	0.39	8.60	58	32.00	3.10	64.00	5.85			
	Dough	11	0.41	8.10	34	36.00	2.92	36.00	2.92			
									8.77	1.09	0.56	7.6

TABLE I—*Continued*

Plant	Dough	Flour brand[1]	Ash[2]	Protein[2]	Viscosity[2,3]	Relative percentage of flours	Relative quantity of protein	Ratio of sponge to dough flours	Relative quantity of protein in sponge, dough, and total flours	Soda per one-barrel batch	Percentage of soda based on flour	pH of the cracker
			%	%		%	%	%	%	*lbs.*	%	
E	Sponge	12	0.39	8.70	52	14.28	1.24					
		13	0.42	8.90	62	28.57	2.54					
		14	0.41	8.70	60	28.57	2.49	71.42	6.27			
	Dough	15	0.54	8.90	26	14.29	1.27					
		16	0.39	8.70	60	14.29	1.24	28.58	2.51			
									8.78	1.30	0.66	7.8
F	Sponge	17	0.45	8.50	40	11.91	1.01					
		18	0.41	8.80	65	35.72	3.14					
		19	0.41	8.80	56	23.81	2.10	71.44	6.25			
	Dough	17	0.45	8.50	40	10.20	1.47					
		20	0.45	8.00	32	18.36	0.87	28.56	2.34			
									8.59	1.07	0.55	7.6
G	Sponge	21	0.38	9.70	77	30.00	2.91					
		22	0.40	9.90	80	30.00	2.97	60.00	5.88			
	Dough	23	0.42	8.80	56	40.00	3.52	40.00	3.52			
									9.40	1.00	0.51	7.4
H	Sponge	21	0.38	9.70	77	30.00	2.91					
		24	0.41	9.60	74	30.00	2.88	60.00	5.79			
	Dough	25	0.42	8.90	58	40.00	3.66	40.00	3.66			
									9.45	1.35	0.69	7.4

TABLE I—*Continued*

Plant	Dough	Flour brand[1]	Ash[2]	Protein[2]	Viscosity[2,3]	Relative percentage of flours	Relative quantity of protein	Ratio of sponge to dough flours	Relative quantity of protein in sponge, dough, and total flours	Soda per one-barrel batch	Percentage of soda based on flour	pH of the cracker
			%	%		%	%	%	%	*lbs.*	%	
	Sponge	26	0.42	9.80	56	13.33	1.31					
		27	0.40	8.50	35	20.00	1.70					
		28	0.43	9.70	60	13.34	1.29					
		29	0.42	9.00	48	13.34	1.20	60.01	5.50			
I												
	Dough	30	0.40	8.50	32	13.33	1.13					
		31	0.41	9.80	58	26.66	2.61	39.99	3.74			
									9.24	1.03	0.53	7.8
	Sponge	32	0.42	9.80	80	28.57	2.80					
		33	0.49	9.90	58	42.86	4.24	71.43	7.04			
J												
	Dough	34	0.47	9.50	70	28.57	2.71	28.57	2.71			
									9.75	1.00	0.51	7.4
	Sponge	35	0.42	8.80	56	23.81	2.10					
		36	0.40	7.80	36	23.81	1.86					
		37	0.41	8.10	33	23.81	1.93	71.43	5.89			
K												
	Dough	38	0.42	8.70	58	16.67	1.45					
		39	0.41	8.20	26	11.90	0.98	28.57	2.43			
									8.32	1.02	0.52	7.8

[1] Flour brands are recorded in code by number rather than by names.
[2] Based on 13.50% moisture.
[3] Based on 5 cc. normal lactic acid reading by the MacMichael viscosimeter.

TABLE II

UNUSUAL CASES OF FLOUR BLENDS (PATENTS, CUT-STRAIGHTS AND CLEARS), BLENDS WITH SPRING WHEAT FLOURS, AND LABORATORY-MADE CRACKERS

Batch	Dough	Flour brand[1]	Ash[2]	Protein[2]	Viscosity[2,3]	Relative percentage of flours	Relative quantity of protein	Ratio of sponge to dough flours	Relative quantity of protein in sponge, dough, and total flours	Soda per one-barrel batch	Percentage of soda based on flour	pH of the cracker	Remarks
			%	%		%	%	%	%	*lbs.*	%		
1	Sponge	40	0.40	8.80	58	50.00	4.40	50.00	4.40				The same brand of flour in the sponge and dough; ratio 50/50.
	Dough	40	0.40	8.80	58	50.00	4.40	50.00	4.40				
									8.80	0.96	0.49	7.4	
2	Sponge	40	0.40	8.80	58	70.00	6.16	70.00	6.16				The same brand of flour as in batch 1 but the ratio is 70/30.
	Dough	40	0.40	8.80	58	30.00	2.64	30.00	2.64				
									8.80	1.20	0.61	7.4	
3	Sponge	41	0.42	9.80	80	50.00	4.90	50.00	4.90				One brand of flour in the sponge and another brand in the dough; ratio 50/50.
	Dough	42	0.40	8.10	42	50.00	4.05	50.00	4.05				
									8.95	1.00	0.51	7.8	
4	Sponge	43	0.39	9.60	75	50.00	4.80	50.00	4.80				The same brand of flour in the sponge and dough blended with another brand in the dough; ratio 50/50.
	Dough	43	0.39	9.60	75	25.00	2.40						
		44	0.40	8.00	38	25.00	2.00	50.00	4.40				
									9.20	0.98	0.50	7.6	
5	Sponge	45	0.43	9.70	76	60.00	5.82	60.00	5.82				Flours very active in fermentation.
	Dough	46	0.42	8.90	58	20.00	1.78						
		47	0.42	8.20	42	20.00	1.64	40.00	3.42				
									9.24	1.50	0.77	7.5	

TABLE II—*Continued*

Batch	Dough	Flour brand[1]	Ash[2]	Protein[2]	Viscosity[2,3]	Relative percentage of flours	Relative quantity of protein	Ratio of sponge to dough flours	Relative quantity of protein in sponge, dough, and total flours	Soda per one-barrel batch	Percentage of soda based on flour	pH of the cracker	Remarks
			%	%		%	%	%	%	*lbs.*	%		
6	Sponge	48	0.44	9.40	65	30.00	2.82						Dough flour too soft.
		49	0.39	8.80	58	30.00	2.64	60.00	5.46				
	Dough	50	0.37	6.70	18	40.00	2.68	40.00	2.68				
									8.14	0.96	0.49	7.4	
7	Sponge	51	0.40	8.20	51	11.90	0.98						Flour blend too soft.
		52	0.38	7.80	29	35.71	2.79	47.61	3.77				
	Dough	53	0.40	8.10	52	28.58	2.31						
		54	0.45	7.60	17	23.81	1.81	52.39	4.12				
									7.89	0.96	0.49	7.8	
8	Sponge	55	0.42	8.40	54	30.00	2.52						Flour blend too soft.
		56	0.40	8.20	48	30.00	2.46	60.00	4.98				
	Dough	57	0.44	7.00	40	40.00	2.80	40.00	2.80				
									7.78	0.91	0.46	7.6	
9	Sponge	58	0.42	9.80	67	28.57	2.80						Flour blend too strong.
		59	0.48	9.90	60	42.86	4.24	71.43	7.04				
	Dough	60	0.50	9.50	60	28.57	2.71	28.57	2.71				
									9.75	0.91	0.46	7.7	
10	Sponge	61	0.43	10.60	92	30.00	3.18						Flour blend too strong.
		62	0.44	10.10	88	30.00	3.03	60.00	6.21				
	Dough	63	0.42	9.70	82	40.00	3.88	40.00	3.88				
									10.09	0.89	0.45	7.5	

TABLE II—*Continued*

Batch	Dough	Flour brand[1]	Ash[2]	Protein[2]	Viscosity[2,3]	Relative percentage of flours	Relative quantity of protein	Ratio of sponge to dough flours	Relative quantity of protein in sponge, dough, and total flours	Soda per one-barrel batch	Percentage of soda based on flour	pH of the cracker	Remarks
			%	%		%	%	%	%	*lbs.*	%		
11	Sponge	64	0.42	9.50	72	30.00	2.85						Blend of clear and patent flours.
		65	0.62	11.10	26	30.00	3.33	60.00	6.18				
	Dough	66	0.41	8.10	35	40.00	3.24	40.00	3.24				
									9.42	1.60	0.82	7.5	
12	Sponge	67	0.65	11.60	36	60.00	6.96	60.00	6.96				Clear in the sponge and patent in the dough.
	Dough	68	0.42	8.20	26	40.00	3.28	40.00	3.28				
									10.24	1.75	0.89	7.5	
13	Sponge	69	0.60	10.80	38	60.00	6.48	60.00	6.48				Clear in the sponge and cut-straight in the dough.
	Dough	70	0.52	9.40	45	40.00	3.76	40.00	3.76				
									10.24	1.70	0.87	7.8	
14	Sponge	67	0.64	11.70	38	60.00	7.02	60.00	7.02				Clear in the sponge and dough; ratio 60/40.
	Dough	71	0.75	9.80	16	40.00	3.92	40.00	3.92				
									10.94	1.90	0.97	7.8	
15	Sponge	67	0.65	11.70	36	70.00	8.19	70.00	8.19				Clear in the sponge and dough; ratio 70/30.
	Dough	72	0.60	11.20	38	30.00	3.47	30.00	3.47				
									11.66	1.96	1.00	7.7	

TABLE II—*Continued*

Batch	Dough	Flour brand[1]	Ash[2]	Protein[2]	Viscosity[2,3]	Relative percentage of flours	Relative quantity of protein	Ratio of sponge to dough flours	Relative quantity of protein in sponge, dough, and total flours	Soda per one-barrel batch	Percentage of soda based on flour	pH of the cracker	Remarks
			%	%		%	%	%	%	*lbs.*	%		
16	Sponge	67	0.65	11.70	36	35.00	4.10						Two clears in the sponge and one in the dough.
		73	0.61	11.30	38	35.00	3.96	70.00	8.06				
	Dough	74	0.72	9.70	18	30.00	2.91	30.00	2.91				
									10.97	1.88	0.96	7.4	
17	Sponge	75	0.42	9.60	72	30.00	2.88						Regular commercial batch for standard 2-inch-size cracker.
		76	0.40	9.50	70	30.00	2.85	60.00	5.73				
	Dough	77	0.40	8.40	30	40.00	3.36	40.00	3.36				
									9.09	1.25	0.64	7.6	
LABORATORY 18	Sponge	75	0.42	9.60	72	30.00	2.88						Same brands of flours and ratio as in batch 17.
		76	0.40	9.50	70	30.00	2.85	60.00	5.73				
	Dough	77	0.40	8.40	30	40.00	3.36	40.00	3.36				
									9.09	—	0.36	7.6	
Blend with 40% spring wheat Flour brands unknown										0.75	0.38	7.8	Not all production data known.

[1] Flour brands are recorded in code by number rather than by names.
[2] Based on 13.50% moisture.
[3] Based on 5 cc. normal lactic acid reading by the MacMichael viscosimeter.

approximate two-inch square size) and the flours used were mostly 90% to 100% patents, although a few cut-straights were used in some cases.

It can be seen from Tables I and II that the flours used varied widely in protein content from as low as less than 7.0% (Table II; batch No. 6) to as high as more than 10.0% (Table II, batch No. 10). The flours were supplied by mills located as far east as New York and Pennsylvania and as far west as Washington and Oregon; however, the major portion was from the midwest (Kansas, Missouri, Indiana, Michigan, Ohio, and Illinois).

It was observed in some plants that the operators were guided by certain rules in selecting and blending the sponge and dough flours. Some favor sponges consisting entirely of strong flours of 9.5% to 10.0% protein (Table I, B, C, J), while others a combination of strong flours of 9.5% to 10.0% protein with softer-type flours of 8.5% to 9.5% protein (Table I, A, D), whereas still others prefer all soft flours of 8.0% to 9.0% protein (Table I, E, F). In the dough some use soft flours (cookie type, Table I, D, F) while others prefer strong flours (sponge type, Table I, A, J) and still others a combination of strong and soft flours (Table I, B, C). In some cases no distinction was made between sponge and dough flours, and accordingly either all strong or all soft flours for the whole batch (Table I, J, K) were used.

Some operators follow the policy of using the same brands of flours all year, and year after year for the purpose of maintaining a uniform product, while others change brands but retain at least one of the sponge flours. With the same thought in mind some operators limit all flours to three or four brands (Table I, B, C), some to as many as five to six (Table I, E, I), and in some cases one to two, keeping the same brand of flour in the sponge and dough (Table I, A; Table II, batches 1 and 2).

Protein

Because more than two flours of various strengths are usually used per batch (a commercial batch consists of about five to six barrels of flour) and also because of the great variation in the ratio of sponge to dough flours, it was found that more attention should be paid to the quantity of protein contributed by each component flour as reported in Tables I and II under "relative quantity of protein."

The relative quantity of protein is derived by reducing the protein content as given by the analysis to the relative percentage of flour; for example: if the protein is 9.5%, and 25% of the flour was used in the batch, then the relative quantity of protein is one-fourth or 2.38%.

Results that are obtained by adding the figures for the sponge, dough, and total flours as reported in Tables I and II under "Relative quantity of protein of the sponge, dough and total flours" prove very valuable for the selection and blending of the cracker flours.

By comparing the flours in each individual plant and then comparing the flours used between the various plants after a period of several years, it was found that the best results could be obtained if the relative quantity of protein contributed by the sponge flours has a range of 5.5% to 6.5% with an average of 6.0% and for the total flours (sponge and dough) 8.5% to 9.5% with an average of 9.0% (Table I, A to I), bearing in mind that all these flours possessed good fermentation and protein qualities. The crackers—tested over a period of years—made with flours of this range are more uniform in quality, and the doughs apparently have good tolerance because they are less susceptible to unfavorable changes in mixing, fermentation, machining, and baking as a result of changes in the new wheat crop flours. However this does not mean that good crackers are produced only from the flours belonging to this protein range, as there are so many other factors in fermentation and baking that may be operative. On the other hand, good crackers can be produced from the flours exceeding this protein range but the cracker doughs made with these flours do not possess good tolerance and they lack uniformity.

It is to be expected that for flours that are fermented properly, the higher the protein content, the more soda is taken up. This is usually true up to a point where the flours are so strong (Table I, J) that this condition becomes reversed, the fermentation is retarded with less acidity being developed, and consequently a lower amount of soda is used as in the case of soft flour (Table I, K).

In testing crackers over a period of years it was observed that crackers made with either too soft or too strong flours (Table II, batches 7 to 10) were scored low because they lacked in flavor which was described as being "flat," "raw," or "floury." With too strong flours (Table II, batches 9 and 10) the cracker bakers experienced difficulties in manufacturing which caused "buckling" in baking, especially if too stiff doughs were used. "Buckling" is well known among cracker bakers because strong cracker flours are more abundant some seasons than the soft flours. On the other hand, with too soft flours the cracker bakers also experienced difficulties in manufacturing which were manifested by "spreading" or "creeping" of the crackers in baking, especially if too soft doughs were used. The term "creeping" is used when the crackers spread so much on the oven shelf that the sheets touch one another.

Ratio of Sponge to Dough Flours

The relative quantity of protein of the sponge flours is also dependent upon the ratio of the sponge to dough flours. If the amount of sponge and dough flour is the same (ratio 50/50) even with the high protein sponge flours (Table II, batches 3 and 4) the relative quantity of protein for the sponge flours falls below 5.0% and with the soft flours even below 4.5% (Table II, batch 1). Such flours take up less soda and consequently the crackers lack in fermentation flavor (are "raw" and "floury" in eating quality). This is especially true in the case of batch No. 7, Table II, where all flours used were very soft and the ratio was even below 50% (47.61%). The best results are obtained if the major part of the flours (60% to 70%) is used in the sponge.

In blending of cracker flours the point of ratio of sponge to dough flours is sometimes overlooked and for that reason variations in quality of crackers occur from year to year.

Soda

It was found by observation for many years that in the well fermented doughs the flours should take up 0.6% soda (based on the flour as 100) with a range of 0.5% to 0.7%. 0.6% corresponds to 1.176 lbs. soda per one-barrel batch, with a range of 0.98 to 1.372 lbs. (Table I, A to K). In a five-barrel batch (a commercial size) there is a range of 4.9 to 6.860 lbs. with an average of 5.88 lbs. If soda is not adjusted the finished cracker would show a wide variation of pH, as low as 5.0 and as high as 9.0. It must be borne in mind that once soda is adjusted all other variables affecting cracker fermentation such as sponge and dough setting temperatures, fermentation time, sponge room temperature, etc., must be kept constant in order that all finished crackers can be brought to the same pH. In our work the pH of the finished crackers was 7.4 to 7.8, in most cases close to 7.6, showing no appreciable effect of the variation of the soda. However if all other variables affecting the cracker fermentation are kept constant, then any variation in soda will be reflected directly in pH.

In unfermented doughs such as hard-tack and some cookie doughs, the flours take up 0.25% to 0.35% of soda, with an average of 0.30%. Usually 0.75 pound of soda per one-barrel batch of hard-tack is sufficient to bring the pH to an alkaline condition regardless of the grade of flour used. In the fermented doughs (for better-grade crackers) nearly double the amount of soda is used to bring the pH to the alkaline side. This indicates that half of the soda is taken up by the natural acidity present in flour, while the other half is taken up by the acidity created in fermentation. Any variation of soda in the fermented

doughs is largely due to the acidity created in fermentation rather than to variation in the initial (natural) acidity because the flours used are mostly unbleached with the pH 6.0 or close to it.

If less than 0.5% soda is used, because of too low or too high protein in the flours, or as a result of improper ratio of sponge to dough, the crackers lack in fermentation flavor. It is generally believed among cracker bakers that the more soda is taken up by the flours, the more tender and better will be the eating quality of the crackers produced, provided good-grade patent flours are used. This is only true if all of the soda added is taken up by the acidity (natural and created in fermentation), because if there is more soda used, but some of it is present as "free" soda, then this excess of soda causes toughening and lack of spring in the finished product, and if the extreme is reached with the pH exceeding 8.1 (by electrometric determination) then there is an off-flavor and very often "soapy" flavor. It is also a known fact that higher alkalinity affects color of crackers, causing quick browning during baking. Very rarely do patent flours of 90% to 100% extraction take up more than 0.7% soda, but flours may occasionally be encountered that are unusually active in fermentation under most favorable plant and climatic conditions (Table II, batch 5), or if buffer salts are added. Usually if more than 0.7% of soda is added, cut-straights and clear flours (Table II, batches 11 to 16) are used, giving the crackers a strong flavor. The clear flours may take up as much as 1.0% of soda (Table II, batch 15), that is about two pounds per one-barrel batch (1.96 lbs. to be exact), due to a greater fermentation rate resulting in the production of more acid. There is a probability that some acidity in fermentation requiring an extra amount of soda is developed due to bacterial activity, especially if lower-grade clear flours are used and if the yeast is as low as 0.1% and the salt below 1.0% (based on the flour as 100).

The same or similar brands of flours may take up as low as 0.5% of soda under one plant's conditions and as high as 0.7% in another plant (Table I, G, H). This means that the soda requirements of flours, sponges, and doughs are largely governed by the plant and climatic conditions. There may be a correlation of behavior between the same or similar brands of flours at different plants; for instance, if a certain flour takes up an increase of soda of from 0.60% to 0.62% at one plant then a similar increase from 0.65% to 0.67% may occur at another plant. The soda requirements of flour are slightly affected from year to year by a change to new-crop flour.

The fact that soda is a good criterion of cracker fermentation can be observed in the case of newly purchased steel fermentation troughs, for when these troughs are first used the fermentation is retarded to

such an extent that the soda requirements of flours may fall as low as 0.4%, which is just about one-third of the normal fermentation, bearing in mind that the soda requirement of unfermented flour is 0.3% and fully fermented flour 0.6%. The soda should be gradually increased, for it may take as long as two weeks before the same amount of soda is reached as was used in the old troughs.

Similar cases are observed in crackers made from blends of spring and soft winter wheat flours, where the soda may fall as low as 0.38%, showing that practically none of the normal cracker fermentation occurred and that the finished product had all of the characteristics of hard-tack (a product made from an unfermented dough). The spring wheat flours not only do not respond to the usual cracker fermentation but also prevent the normal processes of fermentation in the soft winter wheat flours. Practically the same results were obtained in the experimentally made crackers (in the laboratory), showing that the finished product had all the characteristics of hard-tack, particularly lacking in spring, tenderness and flavor, although the flours used (soft winter wheat) and the amount of shortening were the same as in the crackers manufactured in the plant. The amount of soda was almost as low as in hard-tack (Table II, batches 17 and 18).

It is a well known fact that soda cracker fermentation is a very complex problem and the study of soda requirements of flours is only one factor affecting it. There are many other factors that should be studied alone and in combination to determine the fundamental laws underlying the soda cracker fermentation.

Summary

In crackers the primary function of soda is not a leavening action, but rather it is the neutralization of the initial (natural) acidity present in the flour as well as of the acidity created in fermentation.

Soda is not changed chemically in baking.

The soda requirements of flours in unfermented doughs are 0.25% to 0.35%; in fermented doughs (crackers) made with patent flours, 0.5% to 0.7%; with cut-straights and clears the requirements may be as high as 1.0%.

The amount of soda is one of the most important criteria of cracker fermentation, provided the crackers are brought approximately to the same pH (7.6).

Soda requirements of flours are affected slightly in changing from old to new crop flours.

Plant and climatic conditions exert a large effect upon the soda requirements of flours, cracker sponges, and doughs.

Too high and too low protein in flours and flour blends with improper ratio of sponge to dough require less soda. This causes difficulties in manufacturing, and the finished product lacks flavor.

For better-grade crackers (standard two-inch-square size) the relative quantity of protein of 5.5% to 6.5% for the sponge and 8.5% to 9.5% for the total (sponge and dough) flours is recommended.

Soda ("free") causes off flavors if the pH is over 8.1 in the finished product.

Crackers made with a blend of spring and soft winter wheat flours, and experimentally made crackers, have the characteristics of hard-tack (crackers made from an unfermented dough).

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BLEACHING AGENTS VS. POTASSIUM BROMATE IN BAKING WITH DRY MILK SOLIDS [1]

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(Read at the Annual Meeting, May 1940)

The use of bleaching agents is well established in the flour milling industry, as their correct use has been found to improve definitely the baking qualities of many wheat flours. The addition of potassium bromate, an oxidizing agent to most wheat flours, produces significant improvement in baking quality, it has been found. Many workers believe the effects of bleaching agents and of potassium bromate to be similar, although substantiating evidence for this belief has been lacking. One object of the present investigation has been to obtain information that would tend to prove or disprove this belief.

The baking industry during its rapid rise in the past half century has not overlooked the beneficial qualities of milk. As a result dry milk solids is now used extensively in bread-making formulas. Its use introduces problems to the commercial baker, many phases of which are

[1] Contribution No. 71, Department of Milling Industry. Condensed from a thesis submitted to the graduate faculty, Kansas State College, in partial fulfillment of the requirements for the degree of Master of Science.

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not completely understood. Ofelt and Larmour (1940) demonstrated that the use of dry milk solids tends to offset the harmful effect of adding excessive quantities of potassium bromate. In this investigation it was desired to study the effect of dry milk solids when used with flours given varied bleaching treatments or varied quantities of potassium bromate.

Review of Literature

It is not known when milk was first used as an ingredient in bread doughs but it is probable that housewives have included this ingredient in bread for many centuries. Milk in bread was studied from a nutritional viewpoint by Sherman *et al.* (1921), who found that it caused an improvement in growth and reproduction in rats when fed a simplified diet.

St. John and Bailey (1929a) demonstrated that the buffer action of dry milk solids was appreciable, as shown by the initial hydrogen-ion concentration of freshly mixed doughs and by the relative rates of change in the pH values of control and milk-containing doughs. These workers found that production of total carbon dioxide in yeast-leavened doughs was increased when dry milk solids was superimposed on the control formula. In a subsequent paper St. John and Bailey (1929b) observed that one weight unit of water was necessary with each added weight unit of milk to retain the same relative mobility of suspensions of flour, water, and dry milk solids. Skovholt and Bailey (1932) indicated that the inclusion of dry milk solids in dough increased the mixing time necessary to reach maximum plasticity, as shown by the farinograph.

The work of Amidon (1926) appears to be among the earliest dealing with the effects of dry milk solids on the baking quality of flours. This investigator found that dry milk solids produced an increase in loaf volume greater than the corresponding increase in dough weight. Crust color and character, break and shred, crumb color, flavor and taste, and texture were improved with increasing amounts of dry milk solids.

Skovholt and Bailey (1937) reported that dry milk solids had no effect on proteolytic activity. Diastatic activity was retarded by the introduction of dry milk solids, which reduced the hydrogen-ion concentration; gas production was accelerated; activity of the zymase complex was increased as a partial result of an effected reduction of hydrogen-ion concentration. Ofelt and Larmour (1940) observed that 6% of dry milk solids confers a high degree of tolerance to the action of bromate on flours, and gives optimum baking results.

Bailey (1925) presented an excellent review of literature on the effects and uses of various commercial bleaching agents then in use.

Dedrick (1924) stated that too heavy a treatment of certain bleaching agents had been found to affect the strength and quality of the gluten. Overbleaching also tended to affect the color, imparting a yellowish or bluish gray tint to the flour as well as increasing the acidity of the flour. Agene improved the quality and gave greater loaf volume. Novadelox B bleached pure white, matured the gluten favorably, and did not overbleach when excessive amounts were used. An increase in hydrogen-ion concentration of the flour was reported by Micros (1926), working with chlorine as the bleaching agent. Bleaching resulted in a shorter fermentation time, improved texture, and increased volume in the resultant loaf of bread.

According to Balls and Hale (1936) an optimum condition for proteolytic activity in the dough is absolutely necessary for best baking results. If, through overbleaching or overtreatment with chemical improvers, too many of the proteolytic enzymes have been destroyed, tough and unyielding doughs will be obtained, resulting in unsatisfactory bread. Smith (1937) reported that Novadelox had no discernible improving properties upon doughs, although baked loaves in many instances appeared to be improved in texture. Therefore, the question of overbleaching did not arise. Beta Chlora produced bulky and lively doughs, and loaves of better texture, color, and increased volume. With overtreatment by bleaching agents the doughs became short and lacked elasticity. Hanson (1932) found overbleaching with Beta Chlora detrimental to baking results.

Working (1928) explained the action of oxidizing agents on flour as a liberation of phosphatides which in turn reacted favorably upon the gluten. Geddes (1930) suggested that the benefits derived from the use of bromate may arise from the oxidation of certain germ components, presumably the phosphatides, believed to be detrimental. Attention was directed by Geddes and Larmour (1933) to a possible ionic effect by bromate on the gluten proteins whereby the gas-retaining capacities of the gluten were increased. In a series of papers, Jørgensen (1935a, 1935b, 1936) discredited the phosphatide and electrolyte theories and presented considerable data to support his contention that the benefits derived from bromate and other agents that behave in a like manner in bread doughs are the direct outgrowth of protease inhibition.

Methods and Materials

Ten-bushel lots of Tenmarq, Turkey, and Chiefkan wheats were selected on the basis of purity of variety, protein content, and test weight. The protein contents were approximately equal (Table I), thus making the samples desirable for a quality study from a varietal standpoint.

This flour protein range (12.2% to 12.6%) was desirable for the dry milk solids, bleaching, and potassium bromate investigations because it approximates the average range for many commercial flours from wheat grown in Kansas during 1939.

TABLE I

ANALYTICAL DATA FOR TEN-BUSHEL LOTS OF WHEAT AND FLOURS MILLED THEREFROM

Variety	Test weight of wheat	Moisture		Protein[1]		Ash[1]		Baking absorption[1]
		Wheat	Flour	Wheat	Flour	Wheat	Flour	
	lbs.	%	%	%	%	%	%	%
Tenmarq	59.2	10.4	12.7	13.6	12.2	1.234	0.399	61.6
Turkey	56.7	10.5	13.3	13.6	12.5	1.748	.441	60.8
Chiefkan	58.9	11.9	13.3	13.8	12.6	1.852	.412	62.7

[1] Moisture basis 15%.

The wheat samples were milled on the Kansas State College commercial mill. After milling the unbleached flours were placed immediately in a cold room held at approximately 41°F. Special apparatus was constructed to bleach small samples of flour with gas.

Agene, Beta Chlora, and Novadelox were used as bleaching agents in amounts of ½, 1 (full), 1½, 2, 3, and 4 times the bleach used by many commercial mills as normal for straight-grade flour. The amount of bleach added to each sample was based upon the following schedule as the normal of full bleach: Agene, 3.0 g. per barrel of flour (196 pounds); Beta Chlora, 0.75 oz. per barrel of flour; Novadelox, 0.40 oz. per barrel of flour. One-pound samples were bleached and stored in tightly covered cans at room temperature (70°F.) for 10 days. In order to reduce possibilities of error in bleaching, carotene ($C_{40}H_{56}$) determinations were made on the flours by the method of Binnington and Geddes (1939) and photometric apparatus described by Shrewsbury, Kraybill, and Withrow (1938).

The baking formula used was as follows:

Flour	200 g.
Water (distilled)	As required
Yeast	4 g.
Sugar	12 g.
Salt	3 g.
Shortening (hydrogenated vegetable)	6 g.
Malt syrup (120°L.)	0.5 g.
Dry milk solids	As indicated
Potassium bromate	As indicated

When used, dry milk solids was added at the 6% level in accordance with the findings of Ofelt and Larmour (1940). Absorption values for milk-

containing doughs were increased over milk-free doughs 1% for each 1% of dry milk solids used.

Doughs were mixed at 66 rpm. to optimum consistency determined by observation in a Swanson-Working mixer, using a bowl containing four pins. Mixing time and absorption were held constant for each variety. Doughs were divided into two equal portions after mixing, fermented and proofed at 86°F. and 90% relative humidity.

Fermentation was as follows:

First punch after	105 min.
Second punch after	50 min.
Mold after additional	25 min.
Total	180 min.

A National "pup" sheeting roll was used for punching, while molding was done on a Thompson Model A laboratory molder. The loaves were baked at 430°F. for 24 minutes in a specially designed Despatch oven (Finney and Barmore, 1939). Loaf-volume figures are averages of at least four loaves from two bakes on different days. Some mixes were triplicated when necessary. To be considered satisfactory the two loaves obtained from one mix did not differ more than 25 cc. in volume. When the average of loaves from different mixes agreed within 20 cc. it was considered a satisfactorily checked result. Loaves were cut the following morning to grade internal characteristics and to obtain photographs for a permanent record. In calculating the total baking scores the loaf characteristics were weighted after the method of Barmore, Finney, and McCluggage (1940) in the following manner:

$$\text{Loaf volume score} = (\text{actual loaf volume} - 300\text{ cc.}) \times 0.1$$
$$\text{Grain score} = (\text{score on basis of } 100) \times 0.3$$
$$\text{Texture score} = \text{score made on basis of } 22 \text{ points}$$
$$\text{Color score} = (\text{score on basis of } 100) \times 0.1$$

Experimental Data

The optimum bromate requirements for the three flours are given in Table II. The baking results and carotene determinations are shown in Tables III to V and photographs of representative loaves are shown in Figures 1 and 2. Figures 3, 4, and 5 show loaf volumes and carotene values graphically.

TABLE II

OPTIMUM $KBrO_3$ REQUIREMENTS OF THREE FLOURS WHEN BAKED WITH AND WITHOUT 6% DRY MILK SOLIDS (DMS)

	Tenmarq	Turkey	Chiefkan
	mg.	*mg.*	*mg.*
No milk	2	2	2
With DMS	4	6	4

TABLE III

DATA ON TENMARQ FLOURS GIVEN VARIOUS TREATMENTS AND BAKED WITH OR WITHOUT 6% DRY MILK SOLIDS (DMS)

Treatment	Carotene content	No milk		6% DMS	
		Loaf volume	Total baking score	Loaf volume	Total baking score
	ppm.	*cc.*		*cc.*	
NO TREATMENT					
None	1.64	620	80.1	736	92.7
AGENE (*g. per bbl.*)					
1.5	0.64	660	87.1	770	98.2
3.0	0.40	663	86.7	778	101.2
4.5	0.24	666	88.0	792	102.4
6.0	0.20	670	88.9	796	103.2
9.0	0.14	682	89.3	790	105.3
12.0	0.06	690	90.4	802	105.1
BETA CHLORA (*oz. per bbl.*)					
0.38	0.92	633	80.6	750	95.4
0.75	0.68	620	79.8	724	93.2
1.13	0.60	621	81.5	735	94.7
1.50	0.52	634	85.1	735	96.0
2.25	0.51	668	87.8	745	98.5
3.00	0.50	665	86.3	756	100.6
NOVADELOX (*oz. per bbl.*)					
0.20	1.09	618	79.3	738	95.0
0.40	0.75	633	81.9	747	97.5
0.60	0.49	648	83.6	749	98.6
0.80	0.34	655	82.8	757	98.8
1.20	0.20	656	85.2	771	101.7
1.60	0.16	668	89.5	791	104.3
$KBrO_3$ (*mg. per 100 g. flour*)					
1	—	729	96.6	852	106.8
2	—	745	95.9	864	113.2
3	—	723	92.0	870	114.5
4	—	698	90.1	912	119.9
5	—	667	88.3	899	116.2
6	—	650	84.9	858	112.2
7	—	—	—	852	111.8

TABLE IV

DATA ON TURKEY FLOURS GIVEN VARIOUS TREATMENTS AND BAKED WITH OR WITHOUT 6% DRY MILK SOLIDS (DMS)

Treatment	Carotene content	No milk		6% DMS	
		Loaf volume	Total baking score	Loaf volume	Total baking score
	ppm.	*cc.*		*cc.*	
NO TREATMENT					
None	2.55	607	72.8	638	68.9
AGENE (*g. per bbl.*)					
1.5	1.68	607	73.4	650	74.0
3.0	0.97	616	73.7	652	76.5
4.5	0.76	614	74.7	654	78.5
6.0	0.70	620	79.9	665	81.0
9.0	0.54	630	81.9	664	81.8
12.0	0.42	628	82.4	678	86.0
BETA CHLORA (*oz. per bbl.*)					
0.38	1.81	608	72.4	632	71.7
0.75	1.40	618	76.4	622	73.1
1.13	1.24	628	76.4	627	73.5
1.50	1.15	626	76.3	627	76.0
2.25	1.11	630	80.4	626	74.0
3.00	1.07	646	81.9	638	76.0
NOVADELOX (*oz. per bbl.*)					
0.20	1.78	617	74.8	648	72.6
0.40	1.20	616	74.0	655	77.8
0.60	0.81	628	78.9	649	77.2
0.80	0.71	625	77.9	663	80.6
1.20	0.34	635	81.0	657	82.1
1.60	0.23	651	83.9	673	85.1
$KBrO_3$ (*mg. per 100 g. flour*)					
1	—	751	97.6	739	96.8
2	—	840	107.0	788	101.9
3	—	818	104.4	838	109.2
4	—	816	102.3	879	114.3
5	—	762	97.0	915	117.3
6	—	760	95.4	932	119.0
7	—	—	—	884	115.3

TABLE V

DATA ON CHIEFKAN FLOURS GIVEN VARIOUS TREATMENTS AND BAKED WITH OR WITHOUT 6% DRY MILK SOLIDS (DMS)

Treatment	Carotene content	No milk		6% DMS	
		Loaf volume	Total baking score	Loaf volume	Total baking score
	ppm.	*cc.*		*cc.*	
NO TREATMENT					
None	2.07	557	62.1	606	66.4
AGENE (*g. per bbl.*)					
1.5	1.29	580	66.2	635	72.9
3.0	0.79	580	68.6	637	75.5
4.5	0.67	581	70.0	638	76.0
6.0	0.65	591	72.6	639	79.7
9.0	0.61	590	75.9	642	79.7
12.0	0.53	605	79.2	642	80.5
BETA CHLORA (*oz. per bbl.*)					
0.38	1.38	577	66.6	617	69.5
0.75	1.17	587	69.3	624	72.9
1.13	1.15	587	70.7	616	72.1
1.50	1.13	585	72.0	614	74.2
2.25	1.01	595	76.2	615	74.0
3.00	0.96	605	78.2	620	76.2
NOVADELOX (*oz. per bbl.*)					
0.20	1.18	570	66.6	625	73.5
0.40	0.80	575	67.5	624	75.7
0.60	0.51	584	70.2	627	76.1
0.80	0.39	592	71.5	639	78.3
1.20	0.31	608	73.0	640	78.2
1.60	0.30	608	72.2	645	78.7
$KBrO_3$ (*mg. per 100 g. flour*)					
1	—	613	78.3	639	79.8
2	—	652	86.9	685	86.7
3	—	634	86.3	702	94.1
4	—	628	84.6	712	95.7
5	—	618	82.6	712	94.2
6	—	573	79.1	712	95.0
7	—	—	—	712	95.0

Discussion

The data show that the presence of 6% dry milk solids tends to increase the loaf volume. Tolerance to potassium bromate was obtained through the use of dry milk solids. This suggests that dry milk solids in baking act as a buffering agent. Table V and Figures 2 and 5 show that this buffering effect was most noticeable in Chiefkan. Table II indicates that the use of dry milk solids increased the bromate needed for optimum loaf volume and characteristics in all three varieties. The crust color, break and shred, general appearance of the loaf, and grain and texture were improved by the addition of dry milk solids. It was found easier to check duplicate bakes when the formula included dry milk solids, thus giving evidence that more uniform loaves could be obtained through the use of this milk product.

Four times the average bleach was employed experimentally to determine whether such treatment would cause the flour to break down as it does when an excessive amount of potassium bromate is added. A gradual increase in loaf volume and total baking score indicated that the bread was improved by additions of Agene and Novadelox. Beta Chlora gave the only evidence of overbleaching, which occurred with the intermediate treatments. Without dry milk solids this overbleaching was more pronounced. There was evidently more buffering effect from dry milk solids when Beta Chlora was used than with potassium bromate. The fact that more chlorine, which has definite acidic properties, was used in making a normal treatment of Beta Chlora than a four-normal treatment of Agene may account for the difference in the behavior of the two bleaches. Novadelox, not generally considered as a flour improver, was on a par with Agene in aging effect upon the flour. There is no evidence here as to whether this improvement is a direct result of gluten development by Novadelox, or a result of the filler used in Novadelox acting as yeast food, or a combination of both.

The doughs became tighter as the amount of potassium bromate or bleaching agent added was increased, giving evidence that these treatments increased the water absorption. The use of optimum amounts of potassium bromate produced larger loaves and better bread than any bleaching treatment. It appears that "oxidation" by bleaching and "oxidation" by potassium bromate may be of a different type and the terms should not be used synonymously.

The series of flours used in this study provided an excellent basis for variety comparisons. It can be readily seen that bleached Tenmarq without dry milk solids was superior to Chiefkan with or without dry milk solids. This was also true when the optimum amount of potassium bromate was used. The superior gluten quality of Tenmarq as

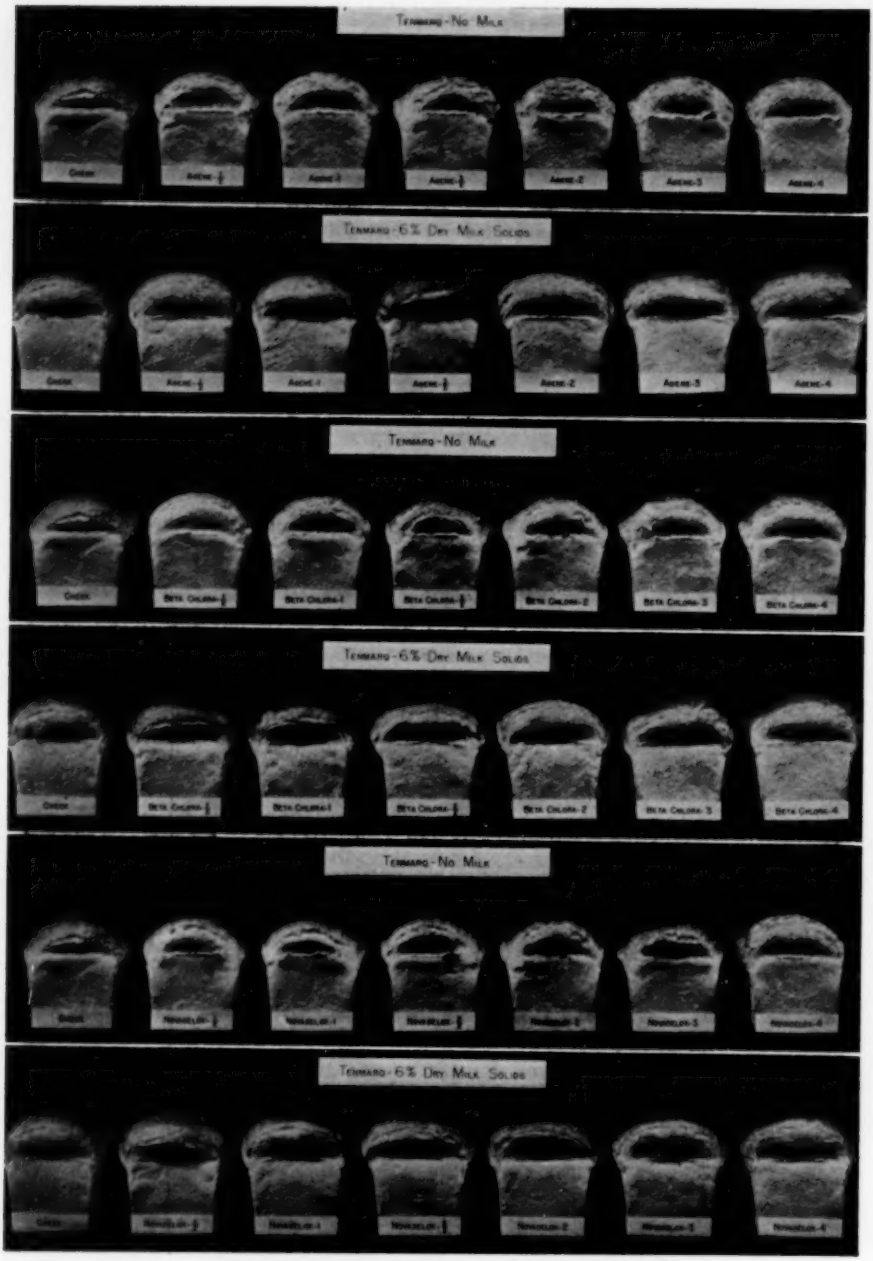
Tenmarq - No Milk
Tenmarq - 6% Dry Milk Solids
Tenmarq - No Milk
Tenmarq - 6% Dry Milk Solids
Tenmarq - No Milk
Tenmarq - 6% Dry Milk Solids

Fig. 1. Bleached Tenmarq flours baked with and without dry milk solids.

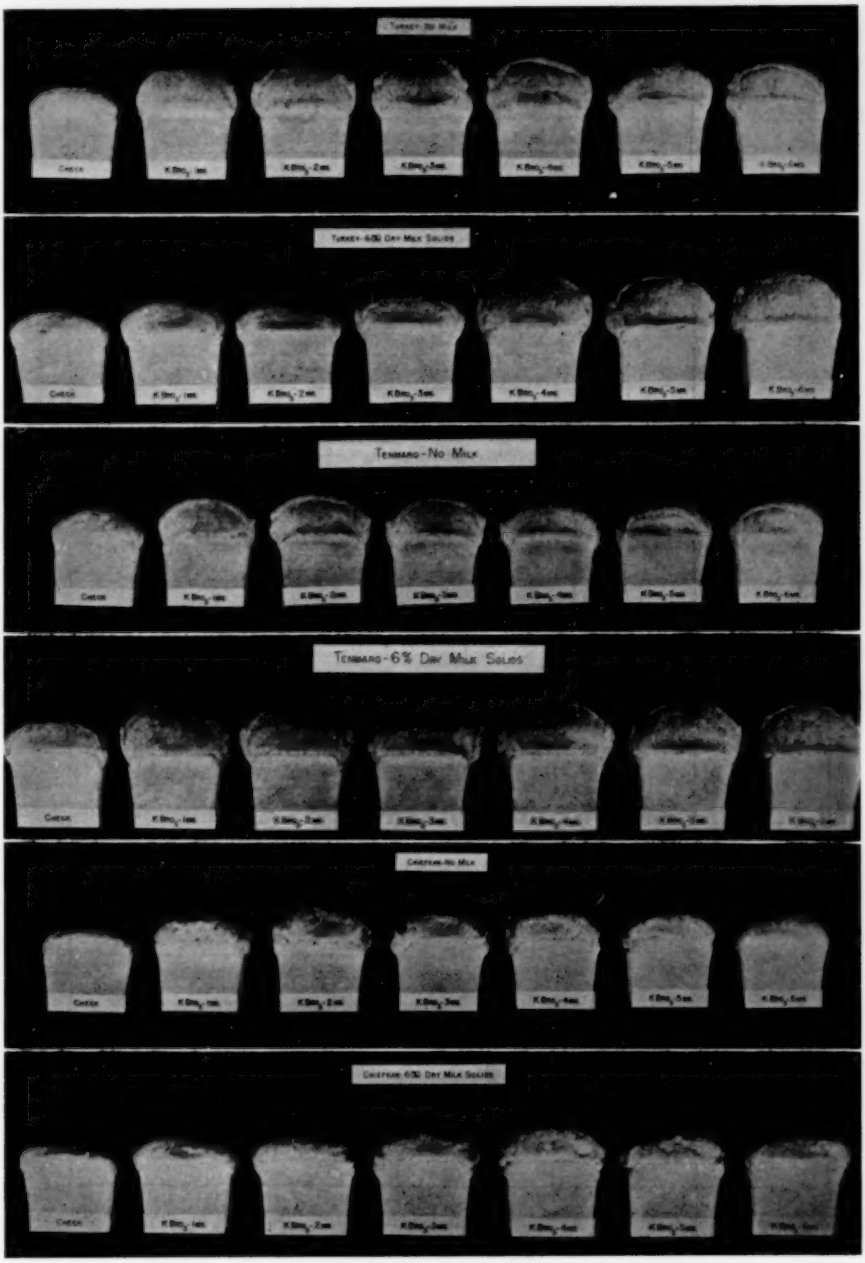

Fig. 2. Loaves baked with various amounts of potassium bromate.

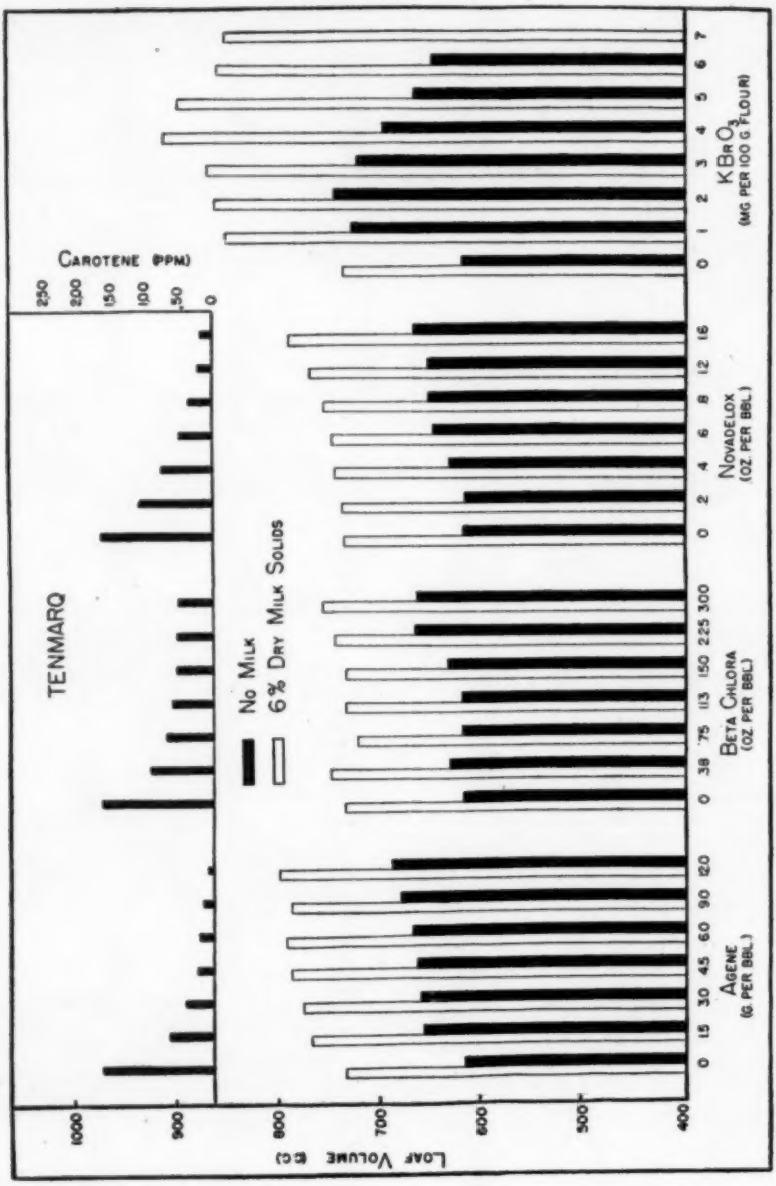
TENMARQ
CAROTENE (PPM)
250 200 150 100 50 0
No Milk
6% Dry Milk Solids
Loaf Volume (cc)
1000 900 800 700 600 500 400
0 15 30 45 60 90 120
AGENE (G. PER BBL.)
0 38 75 113 150 225 300
BETA CHLORA (OZ PER BBL.)
0 2 4 6 8 12 16
NOVADELOX (OZ PER BBL.)
0 1 2 3 4 5 6 7
KBrO3 (MG PER 100 G FLOUR)

Fig. 3. Variations in loaf volume as influenced by bleaching agents, potassium bromate, and dry milk solids. The effect of various kinds and rates of bleach upon carotene content is also shown. Variety of wheat: Tenmarq.

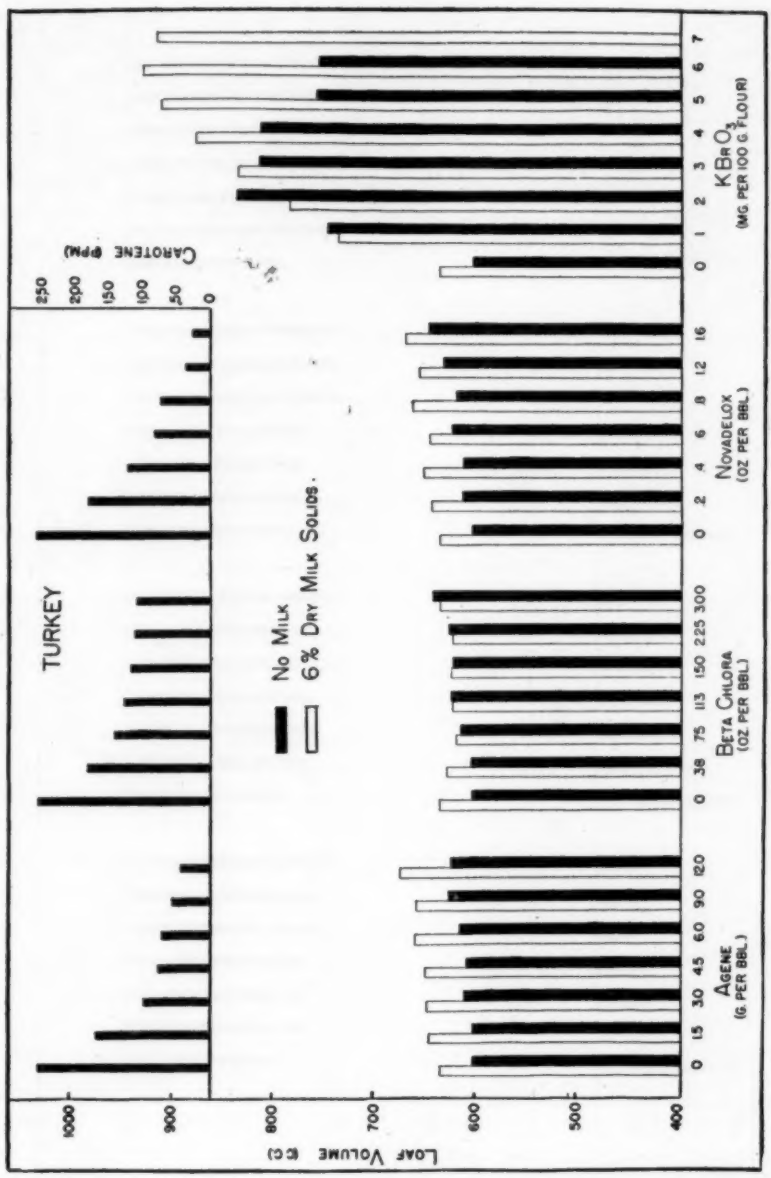

Fig. 4. Variations in loaf volume as influenced by bleaching agents, potassium bromate, and dry milk solids. The effect of various kinds and rates of bleach upon carotene content is also shown. Variety: Turkey.

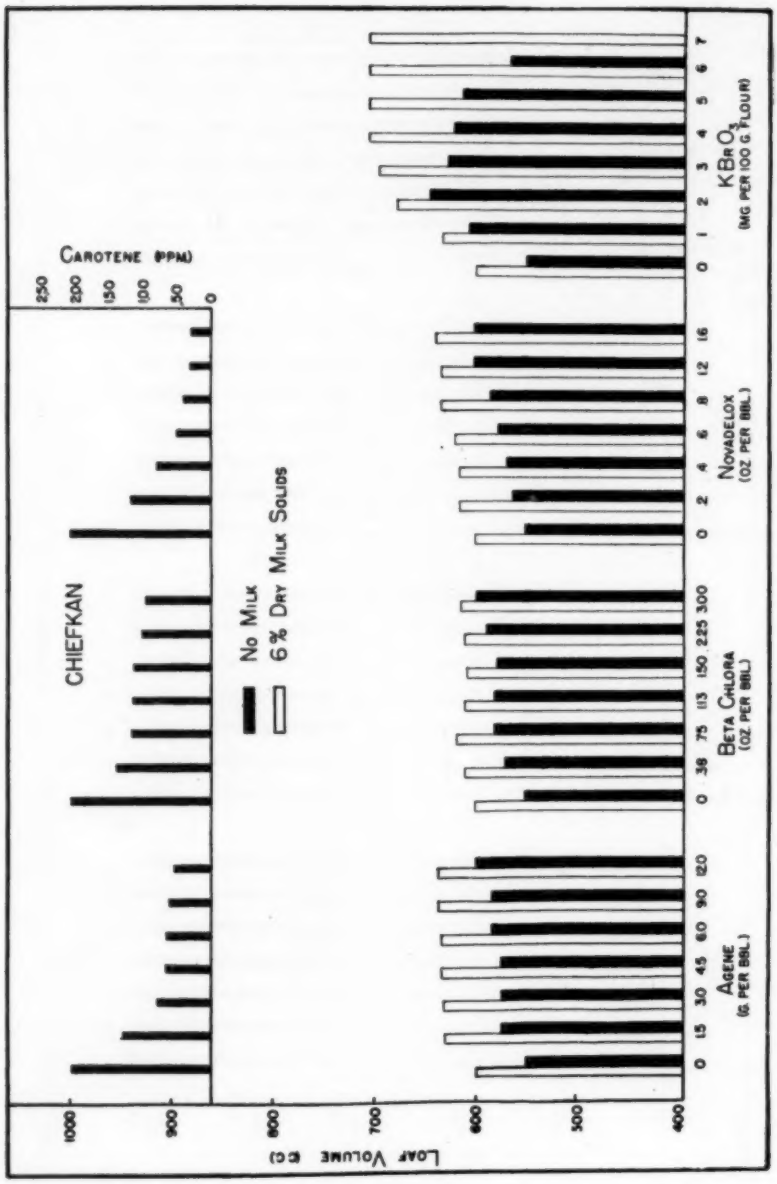

Fig. 5. Variations in loaf volume as influenced by bleaching agents, potassium bromate, and dry milk solids. The effect of various kinds and rates of bleach upon carotene content is also shown. Variety: Chiefkan.

compared to Chiefkan apparently enables it to respond more as a result of adding bromate or dry milk solids in spite of the fact that the Chiefkan sample was higher in protein than the Tenmarq. Turkey definitely had a high requirement for potassium bromate with milk, while Tenmarq did not (Table II). Turkey did not respond relatively as much to bleaching as did Tenmarq. Turkey responded more than Tenmarq to potassium bromate. The latter variety gave better dough development with potassium bromate than with bleaching. Tenmarq definitely displayed the greatest improvement upon the addition of dry milk solids. Chiefkan with dry milk solids possessed a greater tolerance to bromate than any of the varieties investigated. The Tenmarq and Turkey total bread scores at optimum bromate levels with dry milk solids differed only by two points.

Carotene determinations showed that carotene content varied inversely as the amount of bleach applied. These determinations were made to insure the accuracy of results with the bleaching apparatus. Upon this basis the apparatus indicated that it was capable of accurately bleaching one-pound samples of flour with gaseous bleaches.

There was variation in the carotene content of varieties. Unbleached Tenmarq had the least carotene, followed by Chiefkan, while the greatest amount was found in Turkey. However, carotene content decreased relatively the same for all varieties as the amount of bleach increased. As the higher treatments were reached there was a tendency for the carotene content to decrease less rapidly with increasing quantities of bleach. With normal treatments flour and crumb colors were of such a nature that the effect of higher treatments was not easily noticeable.

Beta Chlora was less effective in bleaching the carotene in all flours than was Agene or Novadelox, the latter two being quite comparable in their results.

That the effect of bleaching agents was quite different from that of potassium bromate was evidenced by the increased whiteness in crumb color of bread from bleached flours. Bread made with unbleached flour but with added potassium bromate showed a more yellow color in the crumb than did the bread from bleached flours. This observation further differentiates between bleaching and bromate action and further indicates that the terms should not be used synonymously.

It was evident that flour doughs reacted differently to treatment with bleaching agents and treatment with potassium bromate. Bleaching agents had a notable effect upon the coloring matter of flour, principally carotene, making it colorless. Some effect upon the gluten and baking properties was also evident. The crumb color was whiter, and the volume, grain, and texture were noticeably improved. Potassium bro-

mate, on the other hand, had no appreciable effect upon the coloring matter. It did, however, increase the loaf volume and improve the grain and texture to a much greater extent than did bleaching agents. It is believed from this that potassium bromate acted mainly upon the gluten proteins. This, however, does not eliminate the possibility of its action upon the phosphatides (Working, 1928) nor its inhibitive action upon the proteases (Jørgensen, 1936).

Summary and Conclusions

A study was made of the effects of commercial bleaching agents and of potassium bromate upon the color and baking characteristics of flours from Tenmarq, Turkey, and Chiefkan wheats. In addition, the effect of dry milk solids was studied after the flours were given varied bleaching treatments as well as varied quantities of potassium bromate.

Dry milk solids, when included in the baking formula, was used at the 6% level. The flours were baked with amounts of potassium bromate ranging from 0 to 7 mg. per 100 g. of flour. Agene, Beta Chlora, and Novadelox were the commercial bleaches used in amounts ranging from zero to four times the normal treatment.

The results obtained clearly indicated that the presence of 6% dry milk solids conferred a certain degree of tolerance toward potassium bromate, increased the bromate requirement, increased the loaf volume, and improved the grain, texture, and crust color.

Six percent of dry milk solids increased the loaf volume and improved the grain and texture, crust color, and break and shred of loaves baked from bleached flours.

The bread was gradually improved by the addition of increasing amounts of Agene and Novadelox. Beta Chlora at intermediate treatments gave smaller loaf volumes, with a trend toward increased loaf volumes at heavier treatments.

The carotene content of the flours varied inversely with the amount of bleaching agent used.

The effect of bleaching agents and the oxidizing effect of potassium bromate should not be considered the same, because in no case was the loaf volume or total bread score from bleached flours comparable to that obtained with potassium bromate at the optimum level. Bleaching whitened the crumb color; potassium bromate did not.

Tenmarq and Turkey proved to be superior to Chiefkan in baking quality. Turkey responded the most to potassium bromate. Tenmarq had the least carotene content of the three varieties and responded the most to bleaching treatment as well as to added dry milk solids.

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FACTORS AFFECTING THE SOLUBILITY OF CORN PROTEINS [1]

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(Received for publication December 3, 1940)

As part of a study of corn genetics, it is desirable to analyze for the constituent proteins of the corn kernel. According to Showalter and Carr (1922), Spitzer, Carr, and Epple (1919), and Zeleny (1935), the ratio of the salt, alcohol, and alkali-soluble proteins present in corn varies with the type or strain of corn and the age of the kernel. From the results of a number of analyses it became evident that large variations in the apparent percentages of the different fractions were caused by relatively slight alterations in the procedures used for the extractions. The purpose of this manuscript is to report some of the factors affecting the peptization of corn proteins during such a fractionation.

Since Osborne's early work on the extraction of proteins, evidence has accumulated which shows that the factors determining protein solubility are very diverse and complex. Approaches from the theoretical side, such as those made by Cohn (1938), indicate that the solubility of amino acids (and proteins) in water is influenced by the presence of other amino acids and by salts, with the latter possessing both solvent and salting-out properties, depending upon the nature and the concentration of the salt used.

The extensive investigations of the wheat proteins by Sinclair and Gortner (1933), Blish and Sandstedt (1925), and McCalla and Rose (1935) suggest that the separation of the wheat proteins into fractions soluble in salt, alcohol, and alkali solutions by the Osborne method is largely superficial. Thus most or all of the wheat protein may be peptized by salts such as potassium iodide and sodium salicylate; and, conversely, when the protein is peptized in alcoholic alkali it cannot by neutralization be separated into fractions corresponding to those obtained by direct extraction. Lyotropic studies by Staker and Gortner (1931)

[1] Journal paper No. J-778 of the Iowa Agricultural Experiment Station, Ames, Iowa. Projects 615 and 620. Supported in part by a grant from the American Maize Products Company.

would indicate that the corn proteins are much more sharply differentiated in their solubility than are most of the other cereal proteins.

Anson's recent publication (1939) and Jirgensons' earlier one (1929) show that a lowering of the surface tension of the extraction liquor causes a marked increase in the solubility of a protein, with the solubilized protein becoming denatured. Northrop's work (1939) on the crystalline enzymes indicates that proteins may exhibit a marked tendency to adsorb each other with consequent alteration in their apparent solubility. Work on soybean proteins by Nagel, Becker, and Milner (1938a and b) indicates that solvent extraction and decrease in particle size of the protein-bearing material may increase the amount of protein peptized.

Experimental Methods

Commercial cornmeal, extracted with Skelly A, ground to pass through a 100-mesh sieve, and again extracted, was used for all determinations unless otherwise noted. The salt solution contained 5% NaCl, 0.05 mol of Na_2HPO_4, and 0.01 mol of NaH_2PO_4 (pH about 7.5); the alcohol was 85% ethanol (9 volumes of 95% ethanol plus 1 volume of water); the alkali consisted of aqueous 0.2% NaOH.

Four-gram samples in 200-cc. centrifuge bottles were covered with 100 cc. of the desired solvent and the stoppered bottles rotated longitudinally for specific periods of time (24 hours with salt and alcohol, one to two hours with alkali). The suspensions were centrifuged and the supernatant liquid carefully decanted into Kjeldahl flasks. Each extraction was followed by two 50-cc. washings with the same solvent (except with salt solutions where the second washing was with water), using the same procedure for decantation. Part of the solids in the alkali treatment were gelatinous; in such cases the liquid was carefully siphoned off. Filtration was avoided because it would make difficult successive extractions of one sample with different solvents and because filter paper is known to adsorb proteins (Kingsley, 1940). All extractions were made at room temperature in duplicate or triplicate and usually checked each other within $\pm$ 0.5% of their average. With two exceptions noted below, all experiments were made on the same commercial meal so that the results could be compared.

The preliminary work required to develop methods and technique also served to establish the following facts, which in general confirm similar reports in the literature:

1. Salt and alkali solutions extract proteins rapidly (1 to 4 hours), whereas alcohol acts more slowly. Even after a 24-hour extraction, the values are low unless the alcohol is buffered with sodium acetate.

2. The amount of salt (NaCl) soluble is independent of the salt concentration (5% to 10%) but is increased about 2% by grinding from 60- to 100-mesh and by solvent extraction. An additional increase of 3% may be obtained by continued wet grinding with porcelain balls, although in this case the salt liquor is definitely more cloudy.

3. The volumes of solution as described above are sufficient to remove each of the protein fractions.

Experimental Results

The experimental results have been freed from laboratory details as much as possible and presented in tabular form so that relationships are more apparent. The presentation and tables are arranged according to the following outline:

Table I —The effect of surface active agents.
Table II —The peptizing action of various salts.
Table III—Fractionation by selective solubility.
Table IV—Fractionation by alkali peptization and selective precipitation.
Table V —Effect of pretreatment with alkali.

I—The effect of surface active agents on the solubility of corn proteins is demonstrated by extraction with the sodium salts of organic sulfonates (Table I). It will be noted that the sodium salts of butane sulfonic acid, benzene sulfonic acid, and *p*-phenol sulfonic acid, like sodium chloride, peptized from 18.8% to 20.1% of the protein. The 5% solutions of the larger molecules, which are active wetting agents, peptized from 62.2% to 70.2% of the protein. It should also be noted that the 5% salt solution plus 0.1% Aerosol was less effective as a peptizing agent than either constituent alone.

The butane sulfonic acid salt was made by refluxing butyl bromide and saturated sodium sulfite (in slight excess) in water solution for 30 hours. A fivefold excess of concentrated sulfuric acid, calculated on the sodium bromide, was added, and the solution evaporated repeatedly with the addition of water at 95°C. and under a water pump vacuum until the distillate gave only a faint test for bromide. The sulfuric acid was then removed by neutralization and precipitation from an ice-cold concentrated solution. Evaporation of the filtrate gave a product analyzing about 95% butane sodium sulfonate (by ash and bromide determinations) in 83.5% yield.

II—The peptizing action of various salts, with or without wet grinding, with or without prior extraction of the meal with organic solvents,

TABLE I

EFFECT OF SURFACE ACTIVE AGENTS ON PROTEIN SOLUBILITY—pH 7.5

Reagent	Total nitrogen solubilized
	%
5% buffered NaCl	19.8
1% Aerosol OT [1]	56.7
5% Aerosol OS [2]	63.6
5% sulfonated castor oil [3]	62.2
5% sulfonated olive oil [3]	70.2
5% sulfonated mineral oil [3]	66.8
5% sodium butane sulfonate [4]	18.8
5% sodium benzene sulfonate [4]	19.1
5% sodium *p*-phenol sulfonate [4]	20.1
The meal used in the following experiments was a different brand	
5% buffered NaCl	15.2
0.1% Aerosol OT [1]	13.8
5% NaCl plus 0.1% Aerosol OT [1]	11.8
1% Triton 720 [5]	24.2
0.5% sulfonated olive oil [3]	18.8
0.1% sodium butane sulfonate [4]	6.3

[1] Aerosol OT (American Cyanamid & Chemical Co.) is stated to be the sodium salt of the di-octyl ester of sulfo-succinic acid in 100% purity.

[2] Aerosol OS is stated to be the sodium salt of an alkylated naphthalene sulfonic acid, practically 100% pure.

[3] The sulfonated oils represent products used in the textile industry, and were obtained through the courtesy of H. Rosenberg of the American Finishing Co. of Memphis.

[4] The benzene and phenol sulfonic acids were Eastman products; the butane sulfonic acid salt was made as indicated previously.

[5] Triton 720 (Röhm & Haas) is stated to be an ether sulfonate. The concentration of active material in the sample was not stated, the 1% solution representing 10 cc. diluted to one liter.

lies between 18% and 21% of the total protein of this particular meal. It will be noted that urea is not an effective peptizing agent (Table II).

III—Fractionation of the cornmeal protein by selective solubility has been tested in the following orders: (1) salt, (2) followed by alcohol, (3) followed by alkali, and (1) alcohol, (2) followed by salt, (3) followed by alkali. Prior contact with alcohol decreases the amount of protein peptized by salt and the effect increases with time. The presence of 0.5% sodium acetate in the alcohol increases the amount of protein peptized (Table III).

IV—Fractionation of corn protein by first peptizing the proteins in alkali followed by fractional precipitation gave concordant results for the alcohol fraction only (Table IV). Experiments 1 and 2 represent individual determinations using the procedure as described below directly on the meal. Experiments 3 and 4 are the averages of closely agreeing duplicates in which the salt-soluble fraction was first extracted and the residues then subjected to the above procedure. The value "total soluble" in these latter experiments refers to the amount of nitrogen extracted by the alcoholic alkali and does not include the nitrogen in the salt extracts.

TABLE II

SOLUBILITY OF CORNMEAL PROTEINS IN VARIOUS SALT SOLUTIONS
(Twenty-four hour extraction periods)

Preparation of sample	Reagent	Total nitrogen solubilized
		%
Meal 60-mesh, not solvent-extracted. Porcelain balls present during extraction	5% urea [1]	4
Same	Sodium salicylate [2]	20–21
Same	5% NaCl	20–21
Meal 100-mesh, solvent extracted, no porcelain balls	10% K_2SO_4	18.4
Same	5% NaCl, buffered	18.1
Same	5% sodium butane [3] sulfonate	18.8
Same	5% sodium benzene sulfonate	19.1
Same	5% sodium *p*-phenol sulfonate	20.1

[1] Since urea contains nitrogen the value of 4% was found by difference and is not too accurate, but does indicate that little protein is peptized.
[2] Sodium salicylate of 5%, 7.5%, and 10% concentration all gave similar results.
[3] Compare with values in Table I for sulfonates containing larger radicals.

It will be noted that the amount of alcohol-soluble protein (41.4%–43.0%) by this method of fractionation checks with the value obtained by direct alcohol extraction when the alcohol is buffered with sodium acetate as in Table IV. The amount of protein remaining in the residue is high in comparison with the other procedure and the amount of material remaining in solution (apparently salt-soluble) has little relationship to the proteins actually peptized by salt.

The experimental details for this fraction, modeled after the Blish-Sandstedt (1925) alkali method, are as follows: 4.000-g. samples were

TABLE III

FRACTIONATION OF CORNMEAL PROTEINS BY SELECTIVE SOLUBILITY

Order of extraction	Total nitrogen				
	Salt soluble	Alcohol soluble	Alkali soluble	Insoluble	Recovered
	%	%	%	%	%
Salt-alcohol-alkali	17.4	37.8 [1]	37.1	10.2	102.5
Alcohol-salt-alkali	12.1 [1]	37.6 [1]	37.2	15.6	102.5
Alcohol-salt-alkali	7.1 [2]	43.2 [2]	35.2	11.0	96.5
Using a different brand of meal:					
Salt-alcohol-alkali	15.2	44.2 [3]	27.2	11.6	98.2
Alcohol-salt-alkali	6.6 [3]	45.0 [3]	41.2	10.4	103.2

[1] Alcohol not buffered, 24-hour extraction period.
[2] Alcohol buffered with 0.5% sodium acetate and three extractions, each with 100 cc. of 85% alcohol and for 24 hours, the first removing 41.8%, the second and third 0.8% and 0.6% respectively. The sample was thus in contact with alcohol for 72 hours before the salt extraction as compared with 24 hours in the preceding experiment.
[3] Alcohol buffered with 0.5% sodium acetate, one extraction for 24 hours.

treated with 25 cc. of 0.2% NaOH for five minutes, 75 cc. of 95% alcohol added, and the bottles rolled for one-half hour, after which the liquid was transferred to a 250-cc. volumetric flask by centrifuging and decantation. The solids were again extracted in an identical manner with the same quantities of solvents, and finally washed with 25 cc. of 85% alcohol, after which the volume was made up to 250 cc. with alcohol. A 50-cc. portion was taken for total soluble nitrogen and a 150-cc. portion transferred to a centrifuge bottle and neutralized with 1% HCl

TABLE IV

FRACTIONATION OF CORNMEAL PROTEINS BY ALKALI PEPTIZATION FOLLOWED BY SELECTIVE PRECIPITATION

Experiment	Total nitrogen						
	Salt soluble	App. salt soluble	Alcohol soluble	Alkali ppt.	Total soluble	Insoluble	Recovered
		%	%	%	%	%	%
1	—	13.9	41.4	22.2	77.5	21.8	99.3
2	—	18.9	43.0	22.6	84.5	15.2	99.7
3	19.2	10.6	42.2	4.8	57.6	25.6	102.4
4	18.2	11.4	42.5	4.3	58.2	25.0	101.4

to bromthymol blue. The stoppered bottle was agitated for one to three hours until the precipitate flocculated; then the liquid was centrifuged off and transferred equally to four centrifuge bottles.

The alkali precipitate was washed by centrifuging with a 25-cc. portion of alcohol. The divided liquid was diluted with a total of 400 cc. of 5% NaCl and agitated vigorously until the precipitate (alcohol-soluble) could be centrifuged out completely (about 3 to 4 hours). This precipitate was not coherent as was the first one, so that it was necessary to decant off most of the liquid and separate the last portion by filtering without suction. The precipitate was washed with a small amount of salt solution.

Nitrogen analyses were made upon the original insoluble residue, on the two precipitates denoted as alkali precipitate and alcohol-soluble, as well as for the total amount of nitrogen solubilized. The difference between the total nitrogen solubilized and the sum of the nitrogen values for the two precipitates is denoted as apparent salt soluble.

V—The effect of pretreatment with alkali was investigated by treating the sample with 100 cc. of a 0.2% NaOH solution for one-half hour, neutralizing with acetic acid, then adding solid NaCl to give 5% salt solution, and proceeding with the fractionation by alcohol and alkali. The results in run *A* (Table V) illustrate the effect of adding phenolphthalein indicator to the solution during neutralization. If purified

zein is dissolved in alkali and the solution neutralized in the presence of this indicator a part of the protein fails to precipitate at the neutral point. The precipitated protein also adsorbs some of the indicator. This behavior is common to many proteins according to Hewitt (1927).

The indicator error was avoided in runs *B*, *C*, and *D* by using an outside indicator. It will be noted that the salt-soluble fraction is only slightly depressed and that the alcohol-soluble fraction does not rise to the value of 42% until the alkali-alcohol mixture is used as in Table IV.

TABLE V

EFFECT OF PRETREATMENT WITH ALKALI

Run	Percent of total nitrogen				
	Salt-soluble	Alcohol-soluble	Alkali-soluble	Insoluble	Recovered
	%	%	%	%	%
A	28.3 [1]	21.0	39.5	8.9	97.7
B	18.0	24.0 [2]	43.0 [5]	9.8	94.8 [5]
C	18.4	33.4 [3]	37.6	11.0	100.4
D	16.0	42.4 [4]	—	—	100.4

[1] Value too high due to peptizing action of internal indicator (phenolphthalein).
[2] No buffer added.
[3] Buffered with 0.5% sodium acetate.
[4] After removal of salt-soluble, the residue was treated as in IV.
[5] Low due to analytical loss.

Discussion

Since there are so many factors that modify protein solubility, it is dangerous to make any positive assertions as to which factors are the most important. From the data, it appears that the presence of materials that affect the surface tension of the extracting media and the effect of co-adsorption of proteins are two such factors, important at least in the sense that their effects are difficult to demonstrate, measure, or modify.

In the present work denaturation seems to appear as a factor only when alcohol is the first liquid in contact with the material. Short contact with dilute alkali depresses the solubility in salt and alcohol only slightly.

Since all the non-surface-active sulfonic acids tested behaved as ordinary inorganic salts, whereas all of the detergent sulfonic acids removed large amounts of protein, Anson's conclusion—that the solubilizing effect of such detergents is due solely to a lowering of the surface tension—may be justified. However, neither the present work nor Anson's data are sufficient to disprove an alternate explanation, namely, that the solubilizing effect is due to a chemical interaction between the colloidal protein and the wetting agent. This latter explanation receives

support from the fact that other acids such as the phthaleins and the synthetic tannins, as well as the sulfonated dyes, are known to combine with proteins. The fact that the combination of wetting agent plus salt solubilizes less protein than does either agent alone (Table I) might argue for this latter explanation.

The results shown in Table IV receive a logical explanation if it is assumed that alkali peptization of proteins is influenced by changes analogous to those just discussed under surface tension. The fact that more alkali-soluble protein is removed by a given amount of alkali when the salt-soluble fraction is present than when it is absent would suggest that either nonprotein material present in the salt fraction or the globulin proteins themselves may exert, in alkaline solution, an influence similar to that shown by the surface-active agents in neutral solution. From Table IV it also appears that part of the salt fraction as well as part of the alkali fraction precipitates when the alkali is neutralized, and that not all of the alkali fraction precipitates on neutralization. This receives confirmation in the work of Jones and Csonka (1928), who also have shown that the alkali fraction of corn protein, prepared in a manner essentially that used in experiments No. 1 and No. 2 of Table IV, can be fractionated into two distinct fractions. It would appear probable that one of the fractions they isolated may be (denatured?) globulin. If wheat proteins behave in a manner similar to that of the corn proteins, the facts brought out above would likewise explain why the Blish-Sandstedt alkali method fails to give consistent fractionation for those proteins.

Although none of the experiments were especially designed to show that adsorption is a factor in altering protein solubility, the increased solubilizing action of alcohol containing sodium acetate (Tables III and V) may be explained as due to such a factor. Since adsorption affinities of proteins are known to be dependent upon the pH, the increased solubilizing action caused by the addition of the buffer would be expected if some of the proteins were in an adsorbed condition, and it will be noticed that only when the alcohol is thus buffered do the values for the alcohol-soluble fraction obtained by direct extraction (Table III) check the value found by the selective precipitation method (Table IV). This would indicate that a part of the zein may be adsorbed on the glutenin (or on other material) as it is synthesized by the plant. While the addition of sodium acetate to the alcohol results in an increase in the amount of the alcohol-soluble fraction from meal pretreated with alkali (Table V), the peptization is not complete, as shown by the fact that the check value of 42% may be obtained with the selective precipitation method in which it may be assumed that all adsorption affinities

are nullified. It would seem that precipitation in water solution of a mixture of alkali-peptized zein and glutenin results in a very strong co-adsorption not completely broken by the addition of a buffer to the alcohol.

If the data are viewed in the light of the discussion given above, it is believed that they show that corn proteins do consist of distinct fractions which may be separated upon the basis of differences in solubility behavior. It is seen that by using conditions which minimize the disturbing influences of the factors discussed, it is possible to obtain a salt fraction between 18% and 20% with such different salts as NaCl, K_2SO_4, sodium salicylate, and the three sulfonic acid salts, and to obtain an alcohol-soluble fraction of about 42% by direct extraction, by extraction after removal of the salt-soluble, and by a selective precipitation method. The data do not prove the homogeneity of these three fractions; on the contrary, the salt and alkali fractions would most certainly appear to be mixtures.

Summary

A brief study of the influence of such factors as time, particle size, concentration, and solution volume upon the solubility behavior of corn proteins has been made.

The marked peptizing action of synthetic detergents upon corn proteins has been demonstrated. Non-surface-active sulfonic acid salts were found to peptize only the salt-soluble fraction.

Sodium salicylate was likewise found to peptize only the same amount of protein as do inorganic salts.

Using conditions that minimize the influence of factors altering protein solubility, it was found possible to isolate equal amounts of the various protein fractions by a number of different methods.

It was found that buffered extracting solutions gave more consistent results, presumably by counteracting adsorption.

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AMINO ACIDS IN OATS AND OAT MILLING PRODUCTS, INCLUDING ROLLED OATS [1]

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(Received for publication November 2, 1940)

The justification for the manufacture of a breakfast cereal lies in certain advantages which the manufactured product has over the cereal grain. The product's nutritional value to the consumer should not be sacrificed, however, in obtaining commercial advantages. It is true that breakfast cereals are usually eaten with milk or cream and the addition of fresh fruits is often recommended. It is not likely, therefore, that the daily use of a nutritionally inferior cereal product will lead to a dietary deficiency. Commercializing a product which is inferior to that of the original grain in certain respects, especially in protein, is a poor economy since protein is generally the most expensive dietary factor in our food.

[1] This is the sixth in a series of papers on amino acids in staple foods.

The present investigation on the amino acid content of oats gives information on the quality of oat protein and represents only a phase covered by the term "nutritional value" in which the protein factor, however, is an important one indeed. A sample of rolled oats was compared with the original grain as harvested in respect to amino acid composition in order to establish relative protein nutritional values. There are at least two by-products in the manufacture of rolled oats,[2] shorts and middlings, both of which contain nitrogen and are utilized in commercial feeds. Determination of certain amino acids in these products was also carried out to establish their protein quality values in order to find out whether milling, by causing a separation of different seed components, may also involve a corresponding separation of oat proteins of superior and inferior nutritional values. The actual figures for the amino acids given in Tables II and III are not to be emphasized as they would vary with variety, soil conditions, seasons, etc., but the quantitative changes as produced by the milling process are noteworthy, because in this case a product richer in protein with an improved protein quality is actually obtained.

Experimental

The amino acid determinations were carried out on two samples of oats. One sample was the Richland variety grown at the Iowa Agricultural Experiment Station, Ames, Iowa, in 1938, and the other furnished by a manufacturer, designated in the table as "Commercial." The latter sample, rather rich in protein, harvested in 1938, did not necessarily represent a single variety, but had been cleaned and graded to uniformly large kernels. The samples of "Commercial" oats, rolled oats, and the by-products were all taken by the manufacturer from the same run of oats in "rolled oats" milling. The two samples of oats and the rolled oats were first put through a Hobart grinder and then the meals were further ground in a Wiley mill to obtain a finer product. The middlings and shorts representing the by-products were analyzed as ground by the "rolled oats" mill.

Duplicates of 25-g. quantities were used for the determination of amino acids from each sample. The method of extraction, employing successively 1% sodium chloride extraction, 70% alcohol (two extractions at room temperature and one at 70°C.), and finally acid-alcohol, has been described in a previous publication (1939). With the whole oat kernel the salt extract represented 29% and the alcohol and acid-alcohol 25%, while 40% of the total nitrogen was found in the residue. The extraction technique was not applicable to the rolled oats, oat

[2] The reader is referred to H. J. Brownlee and F. L. Gunderson's article on "Oats and Oat Products," Cereal Chemistry, 15: 257–272 (1938), in which an excellent description is given of the rolled oats milling process.

shorts, and oat middlings. The proteins in them were only slightly soluble in the solvents mentioned; apparently the steam heat and drying applied during the process of manufacture had changed their solubilities. The finely ground meals, therefore, were directly hydrolyzed in 20% hydrochloric acid and their basic amino acids determined, according to the method published previously. Since the basic amino acids do not contribute to humin formation, it is justifiable to disregard the humin nitrogen formed during hydrolysis. The extent of humin nitrogen formation when 20% hydrochloric acid is used for the hydrolysis of oat products is shown in Table I. The

TABLE I

DISTRIBUTION OF NITROGEN IN THE HYDROLYSATE OF OATS AND OAT PRODUCTS EXPRESSED IN PERCENTAGES OF TOTAL NITROGEN

	Filtrate N	Humin N
	%	%
Richland oats	86.6	12.3
Commercial oats	87.2	11.5
Rolled oats	91.7	8.3
Middlings	90.4	10.6
Shorts	77.7	19.7

TABLE II

AMINO ACID CONTENT AND TOTAL NITROGEN IN MOISTURE-FREE MATERIAL OF A SINGLE OAT VARIETY AND A "COMMERCIAL" OAT SAMPLE

	Cystine	Trypto-phane	Tyrosine	Arginine	Histidine	Lysine	Total N
	%	%	%	%	%	%	%
Richland	0.075	0.030	1.38	0.248	0.075	0.206	1.84
Commercial	0.079	0.055	1.03	0.516	0.123	0.186	2.22

nitrogen found in the filtrate after the humin in the hydrolysate was filtered off is also given in the table and expressed in percentages of the total nitrogen present in the sample under analysis. The rather high humin nitrogen formation when oat shorts are hydrolyzed is easily explained by the presence of comparatively large amounts of hull and hairy materials.

The cystine content when given was determined in the hydrolysate of the several extracts and residue obtained by the extraction technique. Tyrosine (1937) and tryptophane (1939) were determined by the Folin-Ciocalteu and the May-Rose methods, respectively, as described in the articles referred to by the present author.

It was observed that the separation of lysine phosphotungstate in the hydrolysate of rolled oats was slow and apparently hindered by the

hydrolytic products of starchy components present in the oat kernel. Direct hydrolysis of oat middlings and shorts showed, for instance, no lysine, which would have been significant, if present. Mainly for this reason, attention was directed to the electrical transport procedure, by which it was hoped to separate lysine quantitatively.

The arginine and histidine were first removed from the acid hydrolysate as their silver salts, and their quantities in rolled oats were determined according to the method previously described. The filtrate was acidified with sulfuric acid and freed from silver by hydrogen sulfide; then by the addition of sufficient sulfuric acid all the barium

TABLE III

AMINO ACID CONTENT AND TOTAL NITROGEN IN MOISTURE-FREE SAMPLES OF "ROLLED OATS" AND OAT BY-PRODUCTS

	Tryptophane	Tyrosine	Arginine	Histidine	Lysine[1]	Total N
	%	%	%	%	%	%
Rolled oats	0.274	0.54	0.724	0.234	0.205	2.95
Middlings	+	1.02	0.814	0.261	0.129	3.44
Shorts	Trace	0.95	0.493	0.101	0.185	2.26

[1] By electrical transport separation.

TABLE IV

PROTEIN QUALITY OF OATS AND OAT MILLING PRODUCTS AS INDICATED BY THE AMOUNT OF AMINO ACID OBTAINED FROM ONE GRAM OF CEREAL NITROGEN WITH CASEIN FOR COMPARISON

	Cystine	Tryptophane	Tyrosine	Arginine	Histidine	Lysine
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Richland	40	16	750	134	40	112
Commercial	35	24	464	232	55	84
Rolled oats	—	93	183	245	79	70
Middlings	—	—	296	236	76	37
Shorts	—	—	420	218	44	82
Casein	20	130	405	236	156	475

was removed. The slightly acid liquid was then concentrated *in vacuo* to drive off the hydrogen sulfide and a large part of nitric acid. This concentrate was then transferred quantitatively into the middle chamber (600-cc. Pyrex beaker) of the electrical transport apparatus. The two other chambers were filled with water at a level slightly higher than that of the middle chamber.

Two specially designed tubes (Fig. 1) were used to connect the middle chamber with the cathode and anode chambers, in which platinum gauze served as the electrodes. The connecting tubes were filled with liquid above the bulb by suction, and electrophoresis was continued for 6 days, changing the content of the cathode chamber

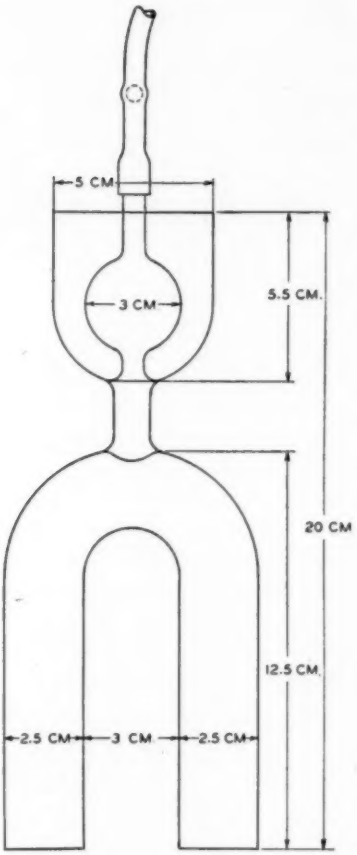

Fig. 1. Connecting vessel.

three times. The combined liquids with washings were concentrated *in vacuo* to a small volume, the ammonia removed, and the lysine precipitated as a phosphotungstate and finally weighed as lysine picrate. The current intensity at the start was set for 30–40 milliamperes, which in the beginning required only 40–50 volts, but toward the end the voltage increased to 300–500 volts. The large width and bulb design on the connecting vessels is to overcome heating. The small vessel that surrounds the bulb is filled with water, the evaporation of which makes cooling even more efficient.

Separation of the three basic amino acids from the hydrolysate by electrical transport gave lower yields for arginine and histidine than by the procedure described above. In following this technique we found lysine in the middlings, although at a lower percentage than that of the "rolled oats."

Discussion

These studies on cereals are intended to obtain information concerning the amino acids of the whole seed kernels with particular emphasis on the indispensable ones, so that we may compare them in respect to quantity with the amino acids of food products manufactured from them. No work has been published from this specific angle relative to the indispensable amino acid content of oats. Quantitative methods suitable for the individual grains had to be devised or selected among those known for the determination of certain amino acids.

As the work has progressed improvements have been added to correct slight errors and to speed up and simplify the technique. Data on more varieties would be desirable to show definite differences in their amino acid percentages. The nitrogen content may serve as a guide in the selection of the proper varieties, but that should not be the final test in judging protein quality. It is shown in Table I that two oat samples may differ in nitrogen content as well as in amino acid composition. The cleaned and graded "Commercial" sample had a higher nitrogen content than the single variety "Richland." It should be noted, however, that by the cleaning and grading process the manufacturer eliminates from "green oats" 25%, more or less, of foreign material and the inferior-sized oat kernels. But the increased nitrogen content of the rolled oats over that of the "Commercial" is only attributable to the milling itself. By removal of the hulls and fuzzy hairs that cover the oat grain the nitrogen content is correspondingly increased. The yield of rolled oats is approximately 40% on the basis of green oats.

The germ, bran, and aleurone layer of the endosperm remain with the rolled oats, but some of the floury endosperm is lost. The increased percentage of tryptophane, arginine, histidine, and lysine in the rolled oats finds its explanation in the elimination of certain low-nitrogen components by the milling process, and at the same time the tyrosine percentage is lowered. The most remarkable change in regard to amino acid composition is found in the by-products. The nitrogen content of oat shorts is similar to that of "Commercial" and the nitrogen content of middlings is even higher than that of rolled oats. The lysine content is low; tryptophane is either absent or, if present, is present only in minute quantities.

The methods of determination were the same in both cases, and there is no known reason to suspect interference with the determination of amino acids in the middlings, which would not have operated in the case of rolled oats. The oat prolamin is known to be free of tryptophane and very low in lysine. Approximately 16% of the total nitro-

gen in oats was found in the 70% alcohol-soluble fraction. Since the protein material in the middlings and shorts originates mainly from the fine floury endosperm of the kernel, the protein in it may chiefly be prolamin. Two extractions of a 10-g. sample of oat middlings with 70% alcohol directly at room temperature followed by one at a temperature of 60°–62°C. showed, however, that only 11.0% of the total nitrogen is alcohol-soluble.

It is still possible that the protein in the middlings consists chiefly of the prolamin type, but by the use of steam heat during the manufacturing process it is largely coagulated, thereby losing partially its alcohol-soluble property. Whatever the correct explanation may be it concerns only the by-products, representing approximately 15% of the cleaned oats used in the manufacture of rolled oats. On the other hand, the favorable change in amino acid composition of "rolled oats" when oats are milled reflects an improved protein quality and the increased nitrogen percentage indicates a product richer in protein.

Summary

Certain amino acids, especially the indispensable ones, were determined in oats, rolled oats (a breakfast cereal), and in two by-products obtained in the milling of rolled oats. It is shown that in eliminating certain components of the oats by the milling process, the rolled oats become richer in protein than the original oat grain and of a better quality nutritionally as judged by the increased percentages of tryptophane, histidine, and lysine. The protein found in the oat by-products, however, is inferior to that present in the whole oat grain.

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ON THE MULTIPLE-AMYLOSE CONCEPT OF STARCH. I. GAMMA-AMYLOSE [1]

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(Received for publication November 8, 1940)

Considerable research in recent years has disregarded the earlier concepts that the various starches are composed of two or more fundamentally different carbohydrates, the latter generally denoted as amyloses. Indeed, so pronounced in the last decade has been the trend away from the multiple-amylose concepts of Maquenne and Roux, Ling and Nanji, and others that the modern tendency is to consider starch as composed of but a single type of dextrose polymer (Armstrong and Armstrong, 1934).

In various attempts by recent workers to explain the incompatibility of this simple picture of starch with the variety of chemical, biochemical, and colloidal phenomena which are not in harmony with it, various theories have been proposed which, for purpose of discussion, have been divided into four groups, since it is beyond the scope of this work to review each individual concept of starch that has been advanced even within the past decade.

The main purpose of the present work is to re-examine these general theories and with further information on one amylose in particular to demonstrate that they are not satisfying—that corn starch at least is composed of two or more distinct chemical entities.

1. The first theory to be discussed proposes that, although the units of starch may be of the same type, *e.g.* linear chains of glycopyranose groups held together by alpha-glucosidic linkages, they are not all of equal chain length. Indeed, some of the more complex chains may have side branches. Thus, the longer ones might be expected to possess different physical characteristics such as solubility, and to exhibit a distinctive behavior with the amylases (Richardson, Higgenbotham, and Farrow, 1935).

2. A second group would explain the major reported differences between various fractions of starch on some physical basis, as for example, in terms of various degrees of dehydration, or of stages of supermolecular aggregation of the primary starch molecules. Reversion or retrogradation are terms commonly used to denote these phenomena (Hirst, Plant, and Wilkinson, 1932; Taylor and Schoch, 1933).

[1] Presented at the 100th Meeting of the American Chemical Society at Detroit, Michigan, September 10, 1940.

3. A third group would attempt to explain the differences previously pointed out for amylose and amylopectin on the basis of noncarbohydrate residues, closely associated with or adsorbed on but not necessarily in combination with, the starch fractions called amylopectin, alpha-amylose, etc.

4. A fourth group would substitute in place of the earlier view—that is, the association of diverse chemical units to form the granule—the chemical union of several radicals into one complex molecule or possibly a branched structure of sufficient complexity to explain the many properties and reactions given by starch. The views advanced by Staudinger and Eilers (1936) and Staudinger (1937), if limited to only one molecule of definite length, and by Freudenberg (1939), for example, might be placed in this category.

It is suggested that a logical organization of existing knowledge of starch particularly as to its biochemical behavior would show that the first and third theories given are no longer attractive. For if, in the first instance, one accepts the theory that starch is a conglomeration of glucopyranose chains of assorted lengths, and if the assumption is made that malt diastase readily attacks only the shortest chains, it seems rather unusual in the light of modern enzyme chemistry (Myrbäck, 1939) that the enzyme should be able to sense, in starting from one end of the chain, whether this terminal is 20 or 30 dextrose units away from the other end (the potential aldose group) or 100 or more units away.

However, granted for the moment that the longest chains are the source of the "limit dextrin" of enzymolysis, it is remarkable, unless in nature there exists a definite plan for producing a certain ratio of long to short chains, that a starch should invariably yield the same ratio of maltose to limit dextrin under a given set of conditions for conversion, and even more remarkable that all of the common starches should yield approximately the same ratio of maltose to residues that are not converted to maltose (Ling and Nanji, 1923; Van Klinkenberg, 1932; Tychowski, 1937).

These latter considerations are as equally unexplainable if, in order to harmonize the theory with the facts found by Haworth *et al.* (1937) and by Hanes (1937) that these unconverted residues are not long chains but relatively quite short (between 6 and 12 glucopyranose units in length), the assumption is made that some of the chains have side branches, which act as a block to further enzymolysis for any chain, long or short.

The constancy of analysis of a starch by diastatic degradation materially weakens the conception of starch as a haphazard conglomeration of assorted molecules. To credit nature with a plan for producing chains that are long and short, or straight and branched, and with a plan

for producing certain amounts of both types for constructing a given starch granule, is to accept the historically basic principle which led to the multiple-amylose concept of starch. By definition (Maquenne and Roux, 1905) the two major fractions discussed above are amylose and amylopectin.

The third theory is undoubtedly the weakest, for first of all it neglects to explain the biochemical differences between starch fractions. Secondly, those who subscribe to it frequently commit an error in logic in advancing their argument to disprove the multiple-amylose concept of starch. They reason from the fact that Taylor distinguished corn alpha-amylose by its combined fatty acid content, while Samec differentiated wheat amylopectin from amylose by its combined phosphorus, and Ling believed combined silica differentiated amylohemicellulose from amylose. Because recent work by Schoch (1938) on corn amylose and on wheat amylose (T. J. Schoch, private communication) and our herein described work on the silica content of corn starch indicate that the non-carbohydrate residues in these cases are possibly impurities, they would then erroneously conclude that the entire multiple-amylose theory collapses, because the basis of distinction between amylose and other amylo-fractions has been shown to be questionable. But even should the observations given above be confirmed beyond question, the conclusion drawn does not follow, because there are other, and it is believed more fundamental, bases of distinction between these amylo-fractions.

To show further that the two theories, discussed above, are erroneously conceived, as well as to collect new information against the second and fourth theories given, which are admittedly less vulnerable on the basis of existing knowledge, were primary objectives of the work herein reported. It is feared that the prevailing failure to take into account the possibility that starch is made up from different types of dextrose polymers might reflect unfavorably on much work that is now being done on the configuration of the starch molecule.

It is intended in this paper, first, to describe an amylose which, for the present, we prefer to call gamma-amylose; second, to show that it is so differentiated from other amyloses as to preclude the possibility that it is a modification of the amylose of Maquenne or Ling or the beta-amylose of Taylor or of Meyer; third, to show its probable relationship to mixtures containing it, but which have been previously described under other names.

Origin of Procedures for Gamma-Amylose Separation

Schruyver, also Ling and Nanji, and others have advanced the hypothesis that certain starches contain a protective coating of a third amylose quite different in its behavior from amylose and amylopectin.

This coating is said to exist on the starches milled from cereals but not from tuber starches. Ling (1928) reported that the material is resistant to the action of barley diastase, by which means it can be separated from the other amyloses that are converted by this treatment into soluble dextrins and sugars. These conclusions have been checked by us and found substantially correct. Whether the whole granule is the smallest botanical unit supplied with a membrane essentially of this material is, we believe, an unsettled question.

Ling called this substance amylohemicellulose. He found no nitrogen in it and believed it to be simply a salt of a silicic acid ester of amylose. That is, its carbohydrate residue was regarded as identical to the soluble amylose which converts quantitatively to maltose.

With the latter facts, our observations are not in accord. The disagreement may be due to a difference between our starch or barley diastase and that used by Ling, or some other uncontrollable factor; otherwise we believe that Ling's observations were in error. Because of this, our product is designated by a new name—gamma-amylose.

Samec (1927) was among the first to take issue with Ling's belief that the silica content of amylohemicellulose is significant. Samec has found that treating the silica-bearing fractions of starch with dilute hydrofluoric acid removes the silica, but that the product is still resistant to enzymolysis and thus differs from the more readily hydrolyzed amylose.

Our product, as isolated, contains only infinitesimal amounts of silica. Indeed, with modern methods of manufacture, producing starch with a minimum of impurities, the total ash of corn starch, for example, is less than 0.1%, of which a considerable part is sodium introduced in processing. The ash of present-day corn starch would have to be all silica, and concentrated exclusively in the 10% fraction that resists barley diastase, if the 10% fraction contained 1% silica, as found by Ling. But these suppositions are not in accord with the analysis of our corn starch, which shows only 0.013% total silica.

Some analytical figures are given for our products in Table I. The first samples (Nos. 1 to 4) of gamma-amylose were isolated from corn starch by procedures given by Ling for amylohemicellulose and quoted by Samec (1927).

These products all showed significant quantities of insoluble protein, indicating that glutinous residues in the starch may have been collected, as might be expected, with the other insolubles after enzymolysis. Inasmuch as our present-day starch is lower in residual gluten than commercial products two decades ago, it is surprising that Ling should have found no nitrogen in this fraction.

Purification of the product was next attempted, and separation of the protein from the amylose with dilute alkali was tried. It was found, however, that this protein dissolved so slowly, and that such relatively high alkali concentration was required, that it would be easier to speedily dissolve out the carbohydrate with alkali, from which solution the relatively insoluble glutinous residue could be centrifuged in the form of a brown sludge. Upon neutralizing the alkaline solution, preparatory to concentration, the observation was made that the product precipitates

TABLE I

PROTEIN, ASH, AND SILICA OF VARIOUS GAMMA-AMYLOSE PREPARATIONS

Preparation number	Protein	Ash	SiO_2
	%	%	%
1	2.97	1.43[1]	0.129
2	2.45	0.55	0.159
3	—	—	0.145
4	2.54	0.50	0.114
5-A	0.24	0.507	0.056
5-B	2.11	0.421	0.170
5-C	0.184	—	—
Sludge (5-B)	32.05	—	—
6-A	0.180	—	—
6-B	0.175	0.201	0.0399

[1] Dialyzed in running tap water, finally in distilled water. All other samples washed with distilled water in centrifuge.

quickly and almost quantitatively, and is again obtainable in insoluble form.

Thus the basis was indicated for experiments to distinguish clearly this product not only from amylopectin and amylose but, what is equally important, from retrograded amylose as well.

Taylor and Morris (1935) have pointed out that corn alpha-amylose is precipitated from alkaline solution by such a neutralization procedure. They showed, moreover, that retrograded beta-amylose dissolves in weak alkali, but is not precipitated on neutralization. From this they argue that their product is not retrograded beta-amylose nor is it contaminated with the latter.

In the experiments to follow it will be seen that Taylor's observation on the behavior of retrograded amylose has been confirmed and that accordingly there is equal justification for the conclusion that our gamma-amylose is chemically and physically different from amylose (Taylor's beta-amylose) or the common physical modification of the latter.

It is appropriate to point out the reasons for our changes in procedure for obtaining the product used in this experiment.

From the above alone it is impossible to distinguish our gamma-amylose from Taylor's alpha-amylose. Indeed, from examination of Taylor's procedures, it would be perfectly logical to conclude that all of the gamma-amylose which had escaped degradation in Taylor's procedures would have concentrated in his alpha-amylose fraction. Taylor has claimed, however, that the reprecipitation of alpha-amylose depends upon its fatty acid content, for in subsequent experiments in this series (Morris, 1935) when the fatty acids are removed from alpha-amylose, he shows the resultant product to remain soluble after neutralization of its alkaline solution.

The first alteration in our procedures was to work with defatted starch. Schoch (1938) has shown that the fatty acids of corn starch may be removed by extraction with the proper fat solvent, and in subsequent experiments, unless specifically indicated otherwise, the starch used for enzymolysis was extracted with dioxane until fat-free, by the method described by Schoch, then thoroughly washed with water before enzymolysis.

To avoid the possibility that incompletely dispersed cellular residues (and hence possibly residues which could have been converted by enzymolysis), whether gamma-amylose or not, would be collected with our insoluble gamma-amylose fraction, the starch was dispersed by solution in alkali before enzymolysis. To further explain the selection of our final procedures for isolating gamma-amylose (as given further on in the "Experimental" section), several products are described as follows:

Product 5–A was prepared by the procedure given, but omitting the final purification of the separated insolubles by dissolving in alkali and reprecipitation by acidification.

Product 5–B was a conversion from the same lot of starch used in 5–A but both the alkali pasting of starch and alkali purification of the final insolubles were omitted. The starch was heat-gelatinized by holding at 90° to 95°C. for 1½ hours prior to enzymolysis. Fifteen grams of product 5–B was soaked for 30 minutes in 150 cc. of water to which was then added at 25°C., 280 cc. of normal NaOH. After 30 minutes of stirring the product was centrifuged and the supernatant liquor decanted from a small amount of brownish sludge. It was acidified to pH 5.0 with HCl and after an hour's standing the precipitate was centrifuged out and further washed and dehydrated as usual. The dry yield recovered was 11.5 g. and was called product 5–C. The alkali-insoluble fraction was neutralized and washed for analysis in similar fashion.

Product 6–B was prepared simultaneously with product 6–A by our finally adopted procedures (as given in the "Experimental" section) except that for 6–B the starch was gelatinized in twice the alkali concentra-

tion normally used. This was to observe any possible advantage of this modification but none was apparent.

The yield of product 6–A was about 5% which is undoubtedly low, due to considerations which follow.

Gamma-Amylose Differentiated from Retrograded Beta-Amylose

Having given the basis for distinction between gamma-amylose and retrograded beta-amylose, we attempted to define more clearly the differences, since admittedly the distinction above discussed is of a quantitative rather than a qualitative nature. It will not be disputed that beta-amylose or its preretrograded form will eventually precipitate after acidification of its alkaline solution.

TABLE II

PRECIPITATE FROM 1% GAMMA-AMYLOSE

	Solutions at		
	pH 3.8	pH 4.4	pH 5.0
	g.	g.	g.
1 hour	0.557	0.492	0.469
1–24 hours	0.235	0.291	0.271
Total	0.792	0.783	0.740

For this purpose more dilute solutions of gamma-amylose, already purified by alkali treatment, were made and studied. Three grams of product 6–A was dissolved in 90 cc. of 0.667*N* NaOH by stirring for 30 minutes at room temperature. It dissolved to a clear solution and was divided into 3 portions of 30 cc. each. To each, 70 cc. of water was added containing enough HCl to adjust the samples to pH 5.0, 4.4, and 3.8 respectively. After one hour's standing each was centrifuged; the supernatant liquor was decanted as quantitatively as possible and allowed to stand an additional 24 hours. A further crop of insolubles was separated by centrifuging. Apparently nothing further separated from the liquors removed after 24 hours' standing, when allowed to stand an additional 24 hours. The insolubles in each case were washed chloride-free with distilled water in the centrifuge and then transferred to an evaporating dish and the dry solids determined. The insolubles resulting from one gram of product are given in Table II.

It would appear that in slightly acid solution the limit of solubility is about 0.2 g. per 100 cc. at 25°C.

In parallel with this experiment, a sample of beta-amylose kindly supplied to us by Dr. T. J. Schoch, prepared by the electromigration of dioxane-extracted autoclaved corn starch was treated in identical manner.

The product swelled slightly in water, but appeared to be rather completely retrograded.

Nothing could be centrifuged at 2400 rpm. in 30 minutes from the beta-amylose solutions within the intervals given, although they did become very slightly opalescent almost as soon as they were neutralized. After the first 24 hours a small but definite amount of material began to flock out of the beta-amylose solutions, more noticeable in the one at pH 3.8. However, even up to 48 hours the quantity was too small to isolate and weigh.

In the section that follows additional proof will be offered that gamma-amylose is not formed by the association of the common amylose units of starch, nor by the obscure mechanism of carbohydrate dehydration, nor by any other process that is in accord with theories commonly used to explain the phenomenon of retrogradation.

Gamma-Amylose as a Product of Synthesis by Diastase

In this section we will deal with the viewpoints of those chemists who, while not necessarily subscribing to theory No. 2, are nevertheless skeptical of separation procedures involving enzymolysis, on the ground that the end products may be the results of synthesis during the course of

TABLE III

DECREASE IN WEIGHT OF INSOLUBLES FROM CORN STARCH AFTER 40 HOURS' DIGESTION WITH BARLEY DIASTASE

Additional hours of conversion	Digestion continued with:	
	Malt diastase	Barley diastase
	g.	g.
0	0.1349	0.1349
68	0.0302	0.0352
120	0.0120	0.0170

the reaction. Synthesis, for example, probably occurs in the extended acid hydrolysis of starch. After sufficient dextrose builds up in solution it is polymerized into various sugars whose configurations may not have existed originally in the starch. The processes are explainable on a physical-chemical basis of equilibria. It was to anticipate this argument that after an initial enzymolysis in our procedures, the maltose, dextrins, and other soluble products of reaction were washed out and our product converted a second time with an excess of barley diastase.

In preparation No. 7, the enzyme treatment was extended after purification of the product by adding fresh enzyme two additional times. In each case there was a gradual decrease in the amount of insoluble gamma-amylose resulting, indicating that the product is slowly hydro-

lyzed and not synthesized by barley diastase. A parallel experiment indicates moreover that the product is digested at a slightly faster rate by an equivalent amount of malt diastase.

The experiment shows, incidentally, that our method is unsuitable for a quantitative estimation of gamma-amylose, since a small part of the product must be solubilized during the course of preparation by barley diastase, especially when alkali-gelatinized starch is used in which the gamma-amylose is evenly dispersed and rendered more accessible to the enzyme.

Returning to the question of synthesis, it is conceivable that a synthesis may result that is not dependent on a reversible equilibrium. For example, the synthesis of the Schardinger dextrins is not a reversible equilibrium inasmuch as we have observed that once these dextrins are formed and isolated, a fresh supply of the amylase from *B. macerans* added to this product does not result in further change. But it is noteworthy that, given the basic products to work with, *B. macerans* is capable of producing the dextrins, whether the source be potato, tapioca, or corn starch.

Thus it seemed advisable to test the synthesis theory for gamma-amylose by allowing barley diastase to work on both tapioca and potato starch. Using our procedures (omitting dioxane extraction of the starch), a yield of only 0.52% was obtained from tapioca, and a yield so small for potato starch, 0.094%, that some doubt exists as to whether gamma-amylose is actually a constituent of potato starch or, rather, an impurity resulting from incomplete removal of some material during milling (Ling, 1928). If " starch molecules " are more or less the same, then it has been shown beyond doubt that gamma-amylose cannot be a product of synthesis. Otherwise, under strictly comparable conditions, substantially the same yields of gamma-amylose would have been obtained from tapioca and potato starch that were obtained from corn starch.

The above experiment also indicates that our product does not result from association of beta-amylose units into an insoluble form, nor is it the end product of any other mechanism used to explain retrogradation. In fact it is apparent from the nature of our procedure that beta-amylose could not be expected to have retrograded during the operations used. We purposely alkali-gelatinized the starch to put into solution any naturally occurring retrograded amylose; we used higher temperatures for conversion (55°C.) than were used by Ling or recommended by Tychowski for barley diastase conversion; and we neutralized our alkali gelatinization only to pH 6.2, thereby providing conditions that are very nearly ideal for keeping starch pastes over long periods of time with a negligible formation of reversion products. This was strikingly con-

firmed in preparing gamma-amylose from tapioca starch. As long as the conditions of our procedures were followed, only very small quantities of insolubles resulted, and these proved to be gamma-amylose. However, in subsequent tests wherein we attempted to shed more light on the meager yield from tapioca starch, we made one conversion at 20°C. The solution, very nearly clear at the start, clouded up considerably in 24 hours, and after several days' standing surprisingly large quantities of insolubles could be separated. This mass, however, proved to be a product of reversion, quite similar to retrograded amylose.

However, some investigators have noted the extent and speed with which corn-starch pastes cloud up on cooling, throwing out apparently more insoluble material than corresponding pastes from potato or tapioca starch, and from the assumption that the above is a retrogradation effect, they may be inclined to propose that the corn-starch molecule, or amylose, is more predisposed to retrogradation than are tapioca or potato amyloses. They should first explain what causes the corn-starch molecule to be more predisposed to retrogradation, if indeed all starch molecules are fundamentally the same. As against the foregoing assumption it is noteworthy that with 5%–10% pastes made by alkali-gelatinizing dioxane-extracted corn, tapioca, and potato starch, the relative cloudiness which immediately results from acidification to pH 5.0 stands in qualitative relationship, at least, to the relative yields of gamma-amylose obtained from these pastes 24 hours later. The fact that this precipitation occurs immediately and abundantly with corn starch argues rather that the factor responsible for cloudiness and precipitation is gamma-amylose, not retrograded beta-amylose. In conclusion, the supposition above given neglects the possibility that in corn starch there exists a component, soluble with difficulty in water at room temperature but whose solubility is materially increased at higher temperatures.

That the phenomenon discussed is not retrogradation is further confirmed by the extreme ease with which these alkali-gelatinized corn-starch pastes are converted by diastase, which we will demonstrate in the second paper of this series, and wherein we differentiate gamma-amylose from amylo-pectin. Thus it is concluded that gamma-amylose is neither a product of reversion nor the product of any other secondary reaction occurring in the presence of barley diastase.

Quantitative Determination of Gamma-Amylose in Corn Starch

Having reported on such properties of gamma-amylose as its solubility and susceptibility to enzyme attack, it is in order to discuss an attempt to determine quantitatively the gamma-amylose content of corn starch.

Fifteen grams of dioxane-extracted corn starch was treated in a 250-cc. centrifuge bottle by a method similar to that used in isolation procedures, except that the enzyme-to-starch ratio was increased 25%. The bottle was observed during enzymolysis for the purpose of noting the first point at which the insolubles could be quantitatively centrifuged out at 2500 rpm. in 20 minutes. This occurred after about 5 hours' conversion time at 50° to 55°C. The insolubles were thoroughly washed in the centrifuge until chloride-free, without transfer from the original bottle. After the third wash water it was necessary to add ethyl alcohol to the water to facilitate the complete centrifuging of the product. After washing, it was quantitatively transferred to an evaporating dish and the dry solids were determined. The centrifugate from the conversion was allowed to stand 24 hours at 5°C. Nothing further was precipitated.

TABLE IV

YIELD OF GAMMA-AMYLOSE FROM CORN STARCH AT VARIOUS CONVERSION TIMES

Conversion time, barley diastase at 50°–55°C.	Yield (dry basis)
hrs.	%
0	(Estimated 9.15)
5	8.09
8	7.55
16	6.12
24	4.97

Similar conversions were made, extending the conversion time to 8, 16, and 24 hours respectively. The percentage of gamma-amylose obtained at these various times of conversion are given in Table IV.

To estimate the amount of gamma-amylose present in corn starch originally, we plotted the logarithms of the yields obtained against conversion times and extrapolated to zero time (Figure 1). The original content by this method was determined as 9.15%. We are inclined to believe therefore that approximately 10% of the corn-starch granule consists of gamma-amylose.

Gamma-Amylose as a Higher Polymer of Amylose

It is the purpose in this section to consider the possibility that there may be modifications of soluble amylose other than those considered, as for example higher orders of polymerization which would account for the greater resistance and lesser solubility of the component separated by us as gamma-amylose. On the basis of this theory, gamma-amylose should be among the highest. Accordingly, the acetyl derivative of the latter was prepared and studied (see " Experimental " section).

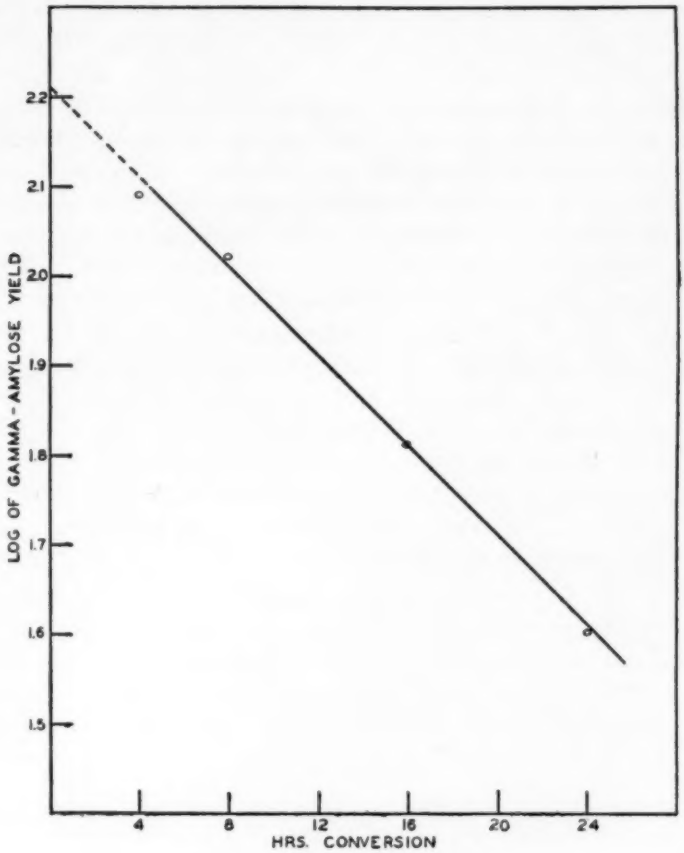

Figure 1.

It may be recalled that the weight of evidence now leads to the conclusion that starch products may be acetylated and deacetylated, under certain conditions, with no change in the degree of polymerization, although Richardson, Higgenbotham, and Farrow (1935) several years ago indicated that this point was controversial. Pringsheim and Persh (1922) and also Pringsheim and Dernikos (1922) pointed out that the Schardinger dextrins from starch could be acetylated in pyridine with acetic anhydride with no change in molecular size, as evidenced by molecular-weight determinations by crysoscopic methods. More recently Staudinger (1937) confirmed the fact that there was no change in the degree of polymerization of various starches during acetylation in pyridine and deacetylation, using osmotic pressure and specific rotation in formamide as a basis of judgment. Finally Higgenbotham and Richardson (1938) adopted the view that the starch molecules remained intact

during acetylation in pyridine since upon deacetylation the product showed the same copper reduction value as the original product before acetylation.

Accordingly, gamma-amylose was acetylated by the acetic anhydride-pyridine method using procedures substantially like those used by Cantor (1934) for acetylation of carbohydrate mixtures. His procedures were selected for the reason that they were shown to involve a minimum of degradation and of rearrangement of the component saccharides (see "Experimental" section).

Acetyl groups, determined by Freudenberg's transacetylation method, were found to be 20.3%, indicating a monacetate. However, the unreliability of this method for the acetates of higher carbohydrates is sug-

TABLE V

Optical Activity of Gamma-Amylose 6-A

Product	Solvent	$(\alpha)_D^{25}$
Potato starch, purified	0.05*N* NaOH	154.0
Gamma-amylose	0.05*N* NaOH	135.0
Gamma-amylose	24 hrs. in 0.05*N* NaOH	135.0
Gamma-amylose	0.10*N* NaOH	134.8
Gamma-amylose	Triton B	135.7
Gamma-amylose	Heated to dissolve in formamid	133.0
Gamma-amylose	Treated as in deacetylation, 0.1*N* NaOH	131.6
Acetyl gamma-amylose	Deacetylated 0.1*N* NaOH	132.5
Maltose (Pfanstiehl)	0.05*N* NaOH	115.0

gested by contrasting this figure with the value obtained by the use of the method of Murray, Staud, and Gray (1931), whereby acetyl groups found were 44.1% and 44.5%, against a theoretical for a triacetate of 44.8%.[2]

A molecular weight of 4,890 ± 390 was found by depression of the freezing point of camphor, which for a triacetate indicates a chain length of 17 ± 2 dextrose units. This result does not substantiate the supposition that gamma-amylose is composed of polymers of a higher order than the commonly occurring amylose units in starch, especially when considered in the light of the estimates of chain length for starch (given in recent years) ranging from 24 to 30 dextrose units (as found by Haworth and Hirst, 1933) up to about 1,000 units (as found by Richardson, Higginbotham, and Farrow, 1936). Indeed, from these modern estimates of the average chain length of starch, the gamma-amylose fraction should be the shortest constituent and could not, therefore, be a polymer of the more frequently occurring beta-amylose.

[2] This analysis was performed by Dr. T. J. Schoch, who has pointed out the unreliability of the Freudenberg method.

Gamma-amylose was deacetylated by prolonged stirring in dilute alkali and the optical rotation determined on the solution at 25°C. For comparison the optical activity of gamma-amylose, 6–A, treated in identical fashion with alkali, is also given in Table V, as well as other pertinent specific rotations.

Several points can be made from the above data.

First, the observation that acetylation procedures such as those used by us involve no perceptible degradation of the carbohydrate can be confirmed. There is, however, a noticeable degradation of the gamma-amylose in prolonged contact with alkali as high as or higher than 0.5*N* even at room temperature, and indeed a measurable one at lower alkalinities if heat is applied (as with formamid). Probably the lower rotation value obtained for gamma-amylose after deacetylation of the acetate is the result of the action of the alkali used in deacetylation.

Secondly, our above-given observation that gamma-amylose is not of greater molecular magnitude than the other more commonly occurring amyloses is confirmed by the fact that the specific rotation of the former is midway between that for maltose and potato starch, the latter starch containing no gamma-amylose.

Third, there is evidence to the effect that our product is uniform. If it is, however, a mixture or series of dextrose polymers (such as a homologous series) rather than one individual molecule, then the variation in molecular size between the highest and lowest members is apparently small.

Gamma-Amylose as a Radical on the Corn Starch Molecule

Those who subscribe to the last of the four theories mentioned early in this paper would doubtless explain the difference between corn starch and potato starch by supposing that gamma-amylose exists in the former (but not in the latter) as a complex radical that is hydrolyzed off by the diastase. But how can they explain the mechanism of this diastatic splitting, since obviously the radical must be attached to one of the ends of the main chain, or to the free end of one of the side chains, or at some intermediate point? The diastase must work gradually up to this point of attachment, rather than to start its hydrolysis by a haphazard attack on some glucosidic chain linkage, which would in all probability form dextrose and maltose, mole for mole. Furthermore, if configurations such as those proposed by Staudinger are to be taken seriously, then rather appreciable amounts of disaccharides other than maltose should also be produced even with beta-amylase. Thus the modern conception is that only the maltose configuration (or some labile configuration that

immediately arranges to maltose after conversion [3]) fits the barley diastases and, in proceeding from a free end, conversion proceeds until a configuration is encountered which is not that of maltose. Hence a branch in the chain or some other anomaly would present a barrier to further enzymolysis as has been suggested by Myrbäck (1939).

If the gamma-amylose radical is attached to the number one carbon of the potential aldose group of the main chain, then for the diastase to reach its point of attachment the chain must be perfectly straight (consist of only maltose groups), and maltose should be the only other product of hydrolysis. The supposition is not granted since the corollary is not in accord with the facts.

If the radical is attached to any other terminal, then the enzyme must start hydrolysis at some other free end which presents a maltose configuration and hydrolysis will continue to the point at which branching of this free end occurs with either the main chain or the branched chain, as the case may be on which it is assumed that the gamma-amylose is attached (granting there are no intervening blocks) and at which point hydrolysis ceases.

If the radical is attached at some intermediate point (*e.g.* carbons 2, 3, or 6 of some intermediate dextrose group) then hydrolysis will proceed from a free end (granting there are no intervening blocks) until the glucopyranose unit to which the gamma-amylose radical is attached is reached and further conversion will cease since a maltose configuration no longer presents itself.

If it be argued in the last two examples that at the point at which hydrolysis ceases the total unconverted residue is in reality gamma-amylose, then some provision must be made to account for the soluble dextrins that are produced during a barley diastase conversion. If it is assumed that these dextrins originate from other starch molecules to which no gamma-amylose radical is attached, then it must be admitted that corn starch consists of at least two fundamentally different types of molecules, the proof of which statement is the thesis of this paper.

Finally, if to avoid accepting the logical conclusions that follow from postulating that gamma-amylose is but a radical attached to a larger starch molecule, one prefers to believe that gamma-amylose is attached to this molecule by a peculiar linkage, and that there exists in barley diastate an enzymatic component capable of splitting this linkage, then it must follow that all starch molecules are not the same. The potato-starch molecule must be constitutionally different from the corn-starch molecule. Tapioca must, in addition, be different from either of these two to account for its lower content of gamma-amylose. Otherwise we

[3] Pringsheim, for example, proposed that the furanose form exists in starch, whereas the third linkage in harmony with methylation data, 1–4, 5 acetal, has recently been considered by Hudson.

must assume that the molecular weight of tapioca starch is ten times as great as that of corn starch, a supposition which would find very little support. Hence, tapioca must contain some carbohydrate molecules like corn—with the gamma-amylose radical attached—and some without. If so much is granted, then the possibility cannot be denied that also within corn starch we might have radically different types of molecules. In this case the necessity for inventing the hypothetical linkage and the hypothetical enzyme to split it vanishes.

Furthermore, in conclusion, it would seem odd that cereal starches such as the corn-starch molecule should be supplied with a resistant barrier were it not to protect the reserve carbohydrate during its formation and storage in close proximity to the embryo. It would seem equally odd that at the same time the embryo, before germination, should be supplied with a special component capable of splitting this barrier from molecules of starch that happened to be exposed. We would rather believe that we have proved that gamma-amylose is a separate and distinct entity quite different from the major constituent, amylose, and that the former is located in the more accessible portions of the granule.

Experimental

Procedures for preparing corn gamma-amylose.—One kilo of commercial cornstarch is extracted with 80% dioxane, preferably in a reflux percolator, until the total fat content is 0.03% or less (according to the method of Sherman, 1932). The starch is quickly air-dried in a forced draft and washed at least three times by suspension in dilute alkali at pH 9. It is then suspended in a small amount of distilled water, passed though No. 20 silk, and made up to 10 liters. Sufficient alkali, either NaOH or KOH, is now added, in solution, to make the resulting paste 0.67 normal in respect to the alkali. It is stirred for 30 minutes at room temperature, whereupon the solution becomes clear and no insoluble starch-cell fragments are observable under the microscope. The solution is now neutralized with stirring for several hours until the paste reaches a pH of 6.2. It should not exceed 13 to 14 liters in volume.

To this paste an extract equivalent to 200 g. of finely ground barley is added, plus 100 cc. toluene. The barley is extracted with about 5 times its weight of cold water, in 2 extractions, the extract precipitated by adding an equal volume of ethyl alcohol and the precipitate redissolved in water and filtered clear.

After 24 hours' conversion at 55°C. the gamma-amylose flocks out and after cooling to room temperature it is centrifuged out and washed in the centrifuge to reduce the solubles. After dilution to approximately 5 or 6 liters and adjustment of pH to 6.2, it is again converted by a repe-

tition of the former treatment, this time with addition of but half the amount of barley diastase used at first.

After separation the second time, the gamma-amylose is washed free of reducing substances. It is then made up to a volume of one liter containing enough potassium hydroxide to make the normality 0.67 and stirred 30 minutes. It is centrifuged and the clear solution neutralized to pH 5.0 with HCl and allowed to stand overnight.

The precipitate is washed chloride-free in the centrifuge, dehydrated by successive washings with absolute ethyl alcohol, washed twice with dry ethyl ether, and dried in a vacuum desiccator.

Acetylation of gamma-amylose.—Ten grams of product was suspended in 62 g. of dry pyridine to which was added, drop by drop, 48 g. of acetic anhydride, with vigorous agitation. The reaction for the first 8 hours was maintained at 5°C. by immersion of the reaction flask in an ice bath. Thereafter for the next 64 hours the reaction was allowed to proceed at room temperature.

During the first few hours the mixture became very viscous and 100 g. more pyridine was added to keep the solution fluid, whereupon 48 g. more of acetic anhydride was added.

After 72 hours' reaction time solution was complete and the reaction was brought to 70°C. and held at that point under a reflux for one hour.

Cooled to room temperature, 250 cc. of $CHCl_3$ was added. The $CHCl_3$ mixture was washed with two 300-cc. portions of 4*N* HCl, then with two 250-cc. portions of dilute Na_2CO_3, and four times with water using 250 cc. each time. The product in $CHCl_3$ solution was then dried by the addition of the excess anhydrous Na_2CO_3, filtered clear, and evaporated to 120 cc. To this solution with vigorous agitation was now added, drop by drop, 150 cc. of petroleum ether. The precipitated mass was filtered dry by suction and washed with two 30-cc. portions of a 2:1 petroleum ether-$CHCl_3$ solution and then with petroleum ether. It was finally suspended in about 150 cc. petroleum ether, filtered and dried *in vacuo*. The yield was 8.5 grams. Found: acetyl groups, 44.1%, 44.5%; molecule weight in camphor, 4,890 ± 390.

Deacetylation of gamma-amylose acetate.—Six hundred and twenty-eight milligrams of the acetate was suspended in 6 cc. of normal NaOH at room temperature. After 30 minutes 5 cc. of H_2O was added and after 24 hours 5 cc. more of normal NaOH. After 48 hours, 25 cc. of water was added, at which point solution was practically complete. After 24 hours more, the solution was made up to 100-cc. volume in a closed volumetric flask and after 24 hours more, with occasional shaking, the optical rotation was determined at 25°C.

Determination of silica.—Gamma-amylose was analyzed for silica by first fusing it with sodium carbonate, leaching the ash first with water,

then with dilute HCl, combining the solubles obtained, and treating an aliquot by the procedures described by Schwartz (1934) and by Swank and Mellon (1934).

Conclusions

Methods for the isolation and characterization of an amylose fraction of corn starch have been presented, which product has been provisionally called gamma-amylose. Tapioca has been found to give but a tenth of the yield given by corn starch, whereas potato starch contains practically none.

Proof has been given that this amylose is different from beta-amylose or physical modifications of the latter, such as would be formed by reversion or retrogradation.

The molecular weight of gamma-amylose acetate is given which indicates that this amylose is a higher polymer of the units that make up beta-amylose.

Evidence has been submitted that gamma-amylose is not a product of synthesis from the intermediate degradation products of starch but exists preformed in the corn-starch granule.

Various theories used to support the modern concept of starch as consisting of but one fundamental type of carbohydrate have been criticized on the basis of the isolation of gamma-amylose and have been shown to be untenable.

The need for a more careful study of the multiple-amylose concept of starch before attempting to determine the configuration of a starch molecule has been pointed out.

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CONVENIENT MEASUREMENT OF GASSING POWER WITH DOUGH UNDER CONSTANT PRESSURE AND WITH ELIMINATION OF GAS SOLUTION

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(Received for publication September 23, 1940)

Many devices for measuring the volume of gas liberated by a dough during fermentation have been described. Common imperfections in the devices have been absorption of the gas by water, varying pressures of the gas whose volume is measured, and inconveniences of manipulation.

In the apparatus of Jago and Kunis (cf. Elion, 1933) the pressure in the space above the water in the displacement vessel is reduced proportionately to the difference in level between the water surface

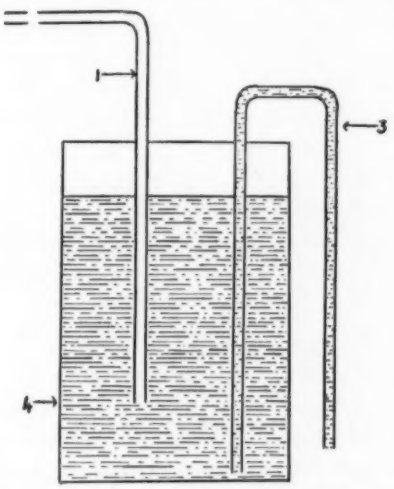

Fig. 1. Irvin's arrangement for constant gas pressure in the dough vessel.

in the vessel and the outer end of the syphon; and, further, the pressure in the dough vessel also is reduced, because the two vessels are in direct connexion. Irvin (1935) described an improved arrangement (Fig. 1), based on "Mariotte's bottle," in which the gas in the dough vessel remains at constant pressure. The inlet tube (1) from the vessel containing the dough is carried almost to the bottom of the water displacement vessel (4), instead of just into the top, as in the apparatus of Jago and Kunis. The length of the outlet tube (3) of the syphon is then adjusted so that with the inlet tube (1) open to the atmosphere, water just fails to drop from the syphon tube (3). This gets rid of

the variation in pressure in the dough chamber, as is shown in Irvin's manometric measurements. The pressure of the gas in the displacement chamber is reduced below atmospheric pressure by the head of water in the syphon—that is, the difference in level between the water level in the displacement chamber and the outer end of the syphon tube (3). The pressure in the dough vessel, however, is balanced at atmospheric pressure by the column of water between the surface of the water in the displacement vessel and the lower end of the inlet tube (1).

Any increase in pressure in the dough vessel causes a gas bubble to escape from the end of the inlet tube (1) up to the surface, where it causes a momentary increase in pressure until conditions are brought back to balance by the flow of water from the syphon. But as the water falls in the displacement chamber, the pressure of the gas in that chamber varies. Thus, the gas is produced under constant pressure but is measured under varying pressure, and Irvin fails to point this out in his description of "a constant pressure gasometer." It is true that the error is unimportant for routine measurements and that a correction can be readily calculated or determined for Irvin's apparatus, because here the corrections depend only upon the dimensions of the displacement vessel. With Jago's apparatus, however, account must also be taken of the errors due to the changes of pressure in the dough jar, and in the volume of the dough jar and its nongaseous contents. Irvin's apparatus also allows interruption of the experiment for dough punching and other manipulations, without the introduction of errors.

The fact that the gas pressure in the dough vessel remains at atmospheric pressure is a distinct advantage when measurements are required of the gas production of dough in large vessels or when the dough vessels vary in volume. Whatever the size of the dough vessel, a given quantity of gas will always displace the same volume of water from the displacement vessel, which would not be true if the gas in the dough vessel were not kept at atmospheric pressure. Irvin's apparatus is also useful if it is desired to keep the dough under normal pressure, as, for example, when measuring the gas production of dough pieces during tin proof. In this case, too, a relatively large container is required to hold the bread tin.

Irvin prevented the solution of the gas by using a salt solution but this does not allow the refilling of the displacement vessel from the thermostat in which it is immersed.

The devices described below possess all the advantages of Irvin's design but, in addition, avoid the use of a salt solution and also allow rapid filling of the apparatus from the thermostat.

Fruit-preserving jars, with metal screw tops and fitted with brass tubes, are very suitable, but either apparatus can be made of metal if desired.

One method is illustrated in Figure 2. Inlet and outlet tubes (1) and (3) correspond exactly to the corresponding ones in Irvin's apparatus but a wide tube (2), open at both ends, surrounds the inlet tube (1). At the bottom of the vessel is a layer of heavy liquid (for example, nitrobenzene or diethyl phthalate) which is not miscible with water and in which the gas is insoluble. When the apparatus is first

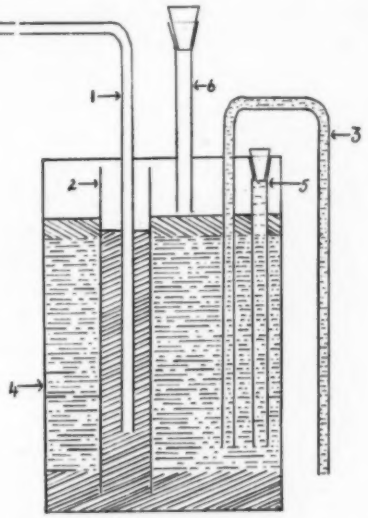

Fig. 2. Arrangement permitting filling of the apparatus from the thermostat, by avoidance of salt solutions.

set up and before any water has been run in, the heavy liquid is introduced. As water is now run in, the heavy liquid rises in tube 2 to a height depending on the water level. It will be seen that the incoming gas will now pass, not through water in which it would dissolve, but through the heavy liquid in tube 2. The end of tube 1 will be under the same static pressure as it would be if water only were used, because since tube 2 is open at both ends, a condition of equilibrium establishes itself. A layer of oil on the surface of the water prevents solution of the gas there.

Refilling takes place through tube 5, the upper end of which is flush with the top of the vessel and opens into the thermostat and the lower end is just clear of the heavy-liquid layer. The water in the thermostat covers the top of tube 5. During refilling, gas and air escape through tube 6, which extends a short distance into the vessel.

When the water rises to the level of the bottom of tube 6, air or gas can no longer escape, so that the water is prevented from rising high enough to flow down tube 2. Tubes 5 and 6 are closed by stoppers during measurements.

The inner leg of the syphon tube (3) ends clear of the heavy-liquid layer, so that there is no danger of this liquid escaping. A dye, Sudan Red, for example, is dissolved in the oil, so as to indicate immediately if the measuring capacity of the vessel is being exceeded.

Both tubes 1 and 6 should extend about four inches above the surface of the water in the thermostat.

The outer leg of the syphon tube (3) is made longer than finally required, and then its correct length determined as follows.

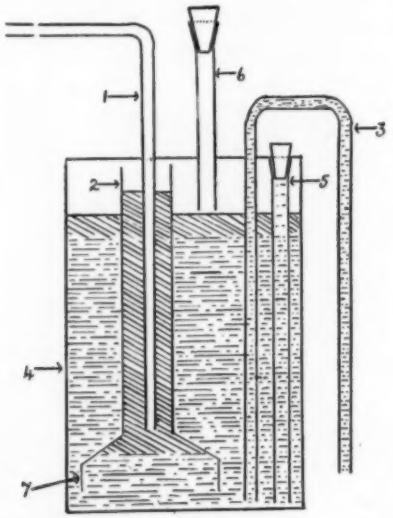

Fig. 3. Alternative arrangement permitting the avoidance of salt solutions.

Fill the vessel by opening tubes 5 and 6; fit a few inches of rubber tubing on to the outlet of the syphon; close 5 and 6 and, with 1 open, start the syphon by gentle suction on the rubber tube. Allow the syphon to run for a short time and then slowly raise the free end of the rubber tube until the flow just ceases. Cut the syphon tube at this level. Minor adjustments can be made by bending the tube so that a drop of water just fails to fall. In practice this is a simple adjustment.

All the manipulations so far described are done once for all when the apparatus is first set up, and they never have to be repeated during routine work.

Routine manipulation.—After setting up and after normal use, the apparatus is left with tube 3 closed by a rubber cap and tubes 5 and 6 open. To begin a measurement, therefore, all that has to be done is to stopper tubes 5 and 6, open tube 3 by removing its cap and, after cessation of flow indicates that equilibrium has been reached, connect the dough vessel on to the inlet tube (1). The evolved gas will now cause water to flow from the syphon tube (3), where it can be collected and measured. At the end of a measurement the dough vessel is disconnected from tube 1, tube 3 is closed and tubes 5 and 6 opened, so that the thermostat water fills the vessel through tube 5.

Alternative method.—Figure 3 shows a method which the writer has used for several years but which is considered inferior to the one just described. A funnel (7) is fitted to the bottom of tube 2 and a space is left between the funnel and the bottom of the vessel. Oil is poured into tube 5 until a layer roughly equal in depth to the height of the funnel is secured. Water is now run in. As the water rises, the oil is entrapped in the funnel and eventually fills tube 2. The volume of the funnel must be equal to or greater than that of tube 2. Tube 6 must be long enough inside the vessel to prevent the water level rising so high as to cause the oil to escape from the top of tube 2.

The manipulation is the same as with the first apparatus.

The gas bubbles up through the oil and, as the water level sinks, the oil receding down tube 2 is retained in the funnel (7). On refilling from the thermostat the oil is automatically returned up tube 2.

In both devices tubes 1 and 6 should be carried well above the water level in the thermostat, and tube 2 should be wide enough to prevent gas bubbles from lifting the liquid upwards. If a rectangular metal cell is used, tube 2 may be formed from a corner cut off by a vertical panel soldered to two adjacent walls of the cell.

Convenient sizes for the tubes are: 1 and 3, $\frac{1}{8}$ inch; 5 and 6, $\frac{1}{4}$ inch; and 2 about $\frac{3}{4}$ inch internal diameter. Tubes 5 and 6 can be fitted with rubber stoppers and tube 3 with a rubber cap.

Summary

It is pointed out that Irvin's gas-measuring apparatus does not measure the gas at constant pressure but does avoid pressure changes in the dough vessel. There follows a description of an apparatus based on Irvin's, in which solution of the gas is prevented by enclosing the inlet tube in another containing a liquid in which the gas is insoluble. This permits the avoidance of salt solutions and hence allows the refilling of the apparatus from the thermostat. Refilling is very rapid and simple.

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